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CHIMERIC PROMOTERS CAPABLE OF MEDIATING GENE EXPRESSION IN PLANTS UPON PATHOGEN INFECTION AND USES THEREOF

The present invention relates to synthetic promoters capable of mediating gene expression in plants upon pathogen infection. The present invention also relates to recombinant genes and vectors comprising said chimeric promoters as well as to host cells transformed with such chimeric promoters, recombinant genes or vectors. The present invention additionally relates to diagnostic compositions and kits comprising such chimeric promoters, recombinant genes, vectors or cells. The present invention also relates to methods for the identification of compounds being capable of activating or inhibiting genes that are specifically expressed in plants upon pathogen infection employing the above described means. Furthermore, the present invention relates to transgenic plant cells, plant tissue and plants containing the above-described chimeric promoters, recombinant genes and vectors as well as to the use of the aforementioned chimeric promoters, recombinant genes, vectors and/or compounds identified by the method of the invention in plant cell and tissue culture, plant breeding and/or agriculture.

The engineering of disease resistance in crops is a major focus of plant biotechnology. One of the most promising approaches to this problem is to engineer defence reactions that are closely related to natural defence mechanisms such as hypersensitive cell death at infection sites, where the cells immediatley surrounding an infection site die in order to prevent further spread of the pathogen (Strittmatter, Bio/Technology 13 (1995), 1085-1089). The controlled generation of highly localised necrotic lesions depends, however, on restricting any cytotoxic activity to the infection sites. This therefore requires promoters that are rapidly and locally responsive to pathogen attack but that also show negligible activity in uninfected tissues.

Initial attempts using large promoter fragments from pathogenesis-related genes such as *prp1-1* have suffered from the disadvantage that it is difficult to isolate a promoter that is totally pathogen specific with substantially no activity in non-infected tissue (Strittmatter, 1995). It seems likely therefore that very few, if any, naturally occuring promoters will be suitable for this purpose.

Recent advances in the detailed study of defence related genes have identified a number of functionally defined *cis*-acting regulatory DNA elements within pathogen inducible promoters (Korfhage, The Plant Cell 6 (1994), 695-708, Raventos, Plant J. 7 (1995), 147-155, Rushton, EMBO J. 15 (1996), 5690-5700). A number of cis-acting elements that are necessary for the response to pathogens have been defined. These include Boxes P and L from the parsley PAL genes (Logemann, Proc. Natl. Acad. Sci. USA 92 (1995), 5905-5909), Boxes H and G from soybean PAL and 4CL (Loake, Proc. Natl. Acad. Sci. USA 89 (1992), 9230-9234), together with a number of less well defined elements. However, while it was shown for a number of such cis-acting elements that they are necessary for elicitor inducibility it was not known whether these elements are sufficient to direct pathogen-induced expression in plant cells and planta on their own. Recently, it has only been shown for the Box W1 from parsley (Rushton, EMBO J. 15 (1996), 5690-5700) and ERE from the maize *Prms* (Raventos, Plant J. 7 (1995), 147-155) that four copies of these elements alone are sufficient to direct elicitor responsive expression to some extent in transient gene expression assays. However, inducibility and background level of expression of the constructs investigated in Rushton, 1996 and Raventos, 1995 greatly varied and at best an about 10-fold induction of reporter gene expression was observed that may not be sufficient to supply the above-described biotechnological needs. Accordingly, it was unclear whether these or any other cis-acting elements may be useful to specifically suppress or confer local gene expression in plants upon pathogen infection.

Thus, the technical problem of the present invention is to provide promoters that are rapidly and locally responsive to pathogen attack but show negligible activity in uninfected parts of the plant and that can be used for engineering of disease resistant crops.

The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the invention relates to a chimeric promoter capable of mediating local gene expression in plants upon pathogen infection comprising

(i) at least one cis-acting element sufficient to direct elicitor-specific expression comprising the nucleotide sequence of any one of SEQ ID NOS: 3 to 16, and

(ii) a minimal promoter.

The term "capable of mediating local gene expression in plants upon pathogen infection" as used herein means that said promoter is capable of controlling the expression of a heterologous DNA sequence at infection sites, analogous or closely related to the controlled expression of pathogen related genes which are involved in the natural resistance in most incompatible host/pathogen interactions, such as the hypersensitive cell death at infection sites of a part of a plant. Thus, the chimeric promoter of the invention is characterized by its capability of mediating localized transcriptional activation selectively in response to pathogen attack or in response to stimuli that mimic pathogen attack such as elicitors prepared from, e.g., pathogens such as fungi or bacteria or derivatives thereof. The transcriptional activation by the chimeric promoter of the invention may also occur in cells surrounding the actual infection site due to cell-cell interactions. The chimeric promoter of the invention may advantageously not or only to a small extent be inducible upon other stimuli such as abiotic stress. Preferably, the induction from the chimeric promoter upon pathogen attack or elicitor treatment is at least about 10-fold higher, preferably 20-fold higher and particularly 30-fold higher than its activation, if any, by abiotic stress.

However, the expression specificity conferred by the chimeric promoters of the invention may not be limited to local gene expression due to pathogens, for example, they may be combined with further regulatory sequences that provide for tissue specific gene expression. The particular expression pattern may also depend on the plant/vector system employed. However, expression of heterologous DNA sequences driven by the chimeric promoters of the invention predominantly occurs

upon pathon infection or treatment with a corresponding elicitor unless certain elements of the invention were taken and designed by the person skilled in the art to control the expression of a heterologous DNA sequence certain cell types.

The term "cis-acting element sufficient to direct elicitor-specific expression" denotes a short stretch of a DNA preferably between 6 and 35 nucleotides in length that when combined with a minimal promoter such as the CaMV 35S minimal promoter (positions –46 to +8) is capable of directing high level elicitor-specific expression of a heterologous DNA sequence. Preferably, said elicitor is a fungal elicitor that can be prepared by conventional means; see, e.g., Ayers, Plant Physiol. 57 (1976), 760-765; Grosskopf, J. Plant Physiol. 138 (1991), 741-746; Kombrink, Plant Physiol. 81 (1986), 216-221; West, Naturwissenschaften 68 (1981), 447-457.

The term "minimal promoter", within the meaning of the present invention refers to nucleotide sequences necessary for transcription initiation, i.e. RNA polymerase binding, and may also include, for example, the TATA box.

The term "pathogen" includes, for example, bacteria, viruses, fungi and protozoa as well as elicitors prepared therefrom.

In accordance with the present invention a number of *cis*-acting elements have been identified that alone are sufficient to direct high level fungal elicitor-specific expression and that can be used to construct novel synthetic promoters that for the first time meet the requirements for engineering disease resistent crops.
Studies that have been performed in accordance with the present invention employed a homologous transient expression system that uses parsley (*Petroselinum crispum*) protoplasts derived from cultured cells. This system is one of very few where the protoplasts respond to fungal elicitor molecules in an almost identical way to cells in the intact plant (Dangl, EMBO J. 6 (1987), 2551-2556; Hahlbrock, Proc. Natl. Acad. Sci. USA 92 (1995), 4150-4157). This allows the study of elicitor-responsive *cis*-acting elements, something that is difficult in many other experimental systems.

Eleven *cis*-acting elicitor-responsive elements (SEQ ID NOS: 3 to 13) were identified in accordance with the present invention. Monomers and multimers of each element were constructed in addition to synthetic promoters consisting of two or more of these elements in combination. Each construct was synthesised with either BamHI ends or with a SpeI site at the 5' end and an XbaI site at the 3' end and then cloned into the corresponding restriction site in front of a minimal CaMV 35S promoter (-46 to +8) in the vector MS23-pBT10-GUS (Sprenger, Ph.D. thesis, University of Köln, Köln, Germany (1997); see Figure 1 (SEQ ID NO: 17) and Figure 2). The distance between the insertion site and the TATA Box varied between 25 and 70bp depending on the insertion site employed and only slight differences, if any, were seen when the same element was inserted into different restriction sites.

Additionally, the cis-acting elicitor-responsive element Box E17 (SEQ ID NO: 15) was identified in accordance with the present invention. Synthetic promoters were constructed comprising a monomer, a dimer or the reverse complement of this element. Various distances between 5 and 131 bp from the inserted Box E17 to the minimal promoter were tested using monomers and dimers (see Example 7). Usable inducibility in the sense of the present invention was obtained for distances of at least 12 bp, and optimal inducibility for distances of 40 to 60 bp to the 5'-end of the minimal promoter.

The experiments performed in accordance with the present invention demonstrate that the *cis*-acting elements direct pathogen-induced expression *in vivo*, being active as monomers, multimers and in combination with each other within synthetic promoters. They therefore meet the biotechnological requirements for the engineering of disease resistance.

In accordance with the present invention these novel chimeric promoters cloned in front of the GUS coding region and the resulting chimeric genes were introduced by means of vacuum infiltration mediated gene transfer into Arabidopsis plants; see Example 6. The expression pattern observed in the transgenic plants containing the GUS marker gene under the control of the chimeric promoter of the invention

revealed expression in tissue infected by bacterial (*Pseudomonas syringae*) as well as by fungal pathogens (*Peronospora parasitica*), whereas local expression in wounded tissues seems to be inactive.

The chimeric promoter of the invention may be preferably comprised only of the above defined cis-acting elements and a minimal promoter. As will be discussed below, other regulatory sequences may be added or present dependent on the intended use of the chimeric promoter of the invention. However, preferably the chimeric promoter of the invention lacks elements that interfere with the elicitor specific expression and/or which are responsible for the non-selective expression of the promoter the cis-acting element of the invention was derived from.

To obtain possible expression in all tissues of a transgenic plant, the minimal regulatory sequences of constitutive promoters are often used, such as the 35 S promoter of CaMV (Odell, Nature 313 (1985), 810-812) or promoters of the polyubiquitin genes of maize (Christensen, Plant Mol. Biol. 18 (1982), 675-689). It is also immediately evident to the person skilled in the art that further regulatory elements may be added to the chimeric sequences of the invention. For example, transcriptional enhancers and/or sequences which allow for further induced expression of the chimeric promoter of the invention may be employed. Enhancer sequences functional in plants include, for example, ocs-element (Ellis, EMBO J. 6 (1987), 3203-3208); the family of ACGT-elements (hex-motif, G-box as 1-element) (Williams, Plant Cell 4 (1992), 485-496) and the cyt-1 element (Neuteboom, Plant J. 4 (1993), 525-534). In order to achieve expression in specific tissues of a transgenic plant it is possible to use tissue specific promoters (see, e.g., Stockhaus, EMBO J. 8 (1989), 2245-2251). Known are also promoters which are specifically active in tubers of potatoes or in seeds of different plants species, such as maize, Vicia, wheat, barley etc. Furthermore, the chemically inducible Tet-system may be employed (Gatz, Mol. Gen. Genet. 227 (1991); 229-237). Further suitable promoters are known to the person skilled in the art and are described, e.g., in Ward (Plant Mol. Biol. 22 (1993), 361-366).

Preferably, the chimeric promoter of the invention further comprises a cis-acting element having the nucleotide sequence of SEQ ID NO: 1 or 2; see Example 5.

In a particularly preferred embodiment of the invention the chimeric promoter comprises homo- and/or hetero-multimeric forms of said cis-acting element(s); see also the appended Example 5. Preferably, said multimeric form is a dimer or tetramer. Particular preferred are those combinations of cis-acting elements that are described in Example 5 and which combination provide for an at least 20-fold, preferably at least 30-fold and particularly preferred at least about 50-fold induction.

In a preferred embodiment of the chimeric promoter of the invention the minimal promoter is derived from the CaMV35S promoter, CHS promoter, PR1 promoter, or hcbt2 promoter. However, other minimal promoters from other sources may be employed as well.

In a further preferred embodiment of the chimeric promoter of the invention, the distance between said cis-acting element and said minimal promoter is 12 to 300 base pairs, more preferably 25 to 70 base pairs, and most preferably 40 to 60 base pairs. In addition or alternatively, a spacer region preferably composed of 4 to 10 base pairs separates at least two of said cis-acting elements in the chimeric promoter. Likewise, it is preferred that at least two of said multimeric forms in the chimeric promoter described above are separated by a spacer of between about 50 to 1000 base pairs.

In a particularly preferred embodiment of the chimeric promoter of the invention the induction of gene expression upon elicitor treatment or pathogen infection is at least 15-fold. As discussed before, the cis-acting elements so far investigated in the prior art only provided for induction upon elicitor treatment of about 10-fold. However, a 10-fold induction of a recombinant gene encoding, e.g., an anti-viral protein may not be sufficient to rapidly and efficiently combat against the pathogen. The present invention provides several cis-acting elements that are capable of inducing high level expression of a given DNA sequence up to 400-fold induction; see, e.g., Example 1.

Furthermore, the invention demonstrates that the combination of otherwise weak cis-acting elements can provide for a substantial increase of the overall inducibility of the chimeric promoter; see Example 5. Thus, the present invention for the first time provides a generally applicable method for how to construct and use chimeric promoters in the field of plant biotechnology. As will be noted from the appended Examples, the background value of the chimeric promoters of the invention may vary to a certain extent. The person skilled in the art therefore may employ different chimeric promoters with different background levels and inducibility depending on the intended use. For example, if the approach of coat protein-mediated protection against virus infection is used the chimeric promoter employed may have high background level expression that would not harm the plant and which upon viral infection would increase at high levels such that resistance to the virus can be obtained. The same rational would apply to, e.g., an antisense or ribozyme mediated protection or the engineering of resistance to fungal pathogens by the expression of anti-fungal proteins etc. On the other hand, where the generation of race-specific resistant genes and artificial generation of hypersensitive cell death is intended, preferably a chimeric promoter is used that has low or substantially no background activity and that only upon pathogen attack is activated to an extent that sufficient level of toxic protein is made so as to cause the cell to die. The selection of the appropriate chimeric promoter of the invention depending on its use is well within the skill of the person skilled in the art.

Examples of the different possible applications of the chimeric promoter according to the invention as well as its cis-acting elements will be described in detail in the following.

Hence, in a further embodiment, the present invention relates to a recombinant gene comprising the above-described chimeric promoter. Preferably, the recombinant gene is configured such that the chimeric promoter is operatively linked to a heterologous DNA sequence.

The term "heterologous" with respect to the DNA sequence being operatively linked to the chimeric promoter of the invention means that said DNA sequence is not naturally linked to the chimeric promoter of the invention.

The term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. The chimeric promoter "operably linked" to a heterologous DNA sequence is ligated in such a way that expression of a coding sequence is achieved under conditions compatible with the control sequences. Expression comprises transcription of the heterologous DNA sequence preferably into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic, i.e. plant cells are well known to those skilled in the art. In the case of eukaryotic cells they comprise optionally poly-A signals ensuring termination of transcription and stabilization of the transcript, for example, those of the 35S RNA from Cauliflower Mosaic Virus (CaMV) and the Nopaline Synthase gene from Agrobacterium tumefaciens. Additional regulatory elements may include transcriptional as well as translational enhancers. A plant translational enhancer often used is the CAMV omega sequences, the inclusion of an intron (Intron-1 from the Shrunken gene of maize, for example) has been shown to increase expression levels by up to 100-fold. (Mait, Transgenic Research 6 (1997), 143-156; Ni, Plant Journal 7 (1995), 661-676). In this respect, it should be noted that in one embodiment of the recombinant gene of the invention at least one of said cis-acting elements is located in the 5'- or 3-untranslated region or in an intron of the recombinant gene.

In a preferred embodiment of the recombinant gene of the invention said heterologous DNA sequence encodes a (poly)peptide, cytotoxic protein, antibody, antisense RNA, sense RNA, ribozyme, transcription factor, protease, nuclease, lipase, or polymerase

The recombinant gene of the invention can be used alone or as part of a vector to express heterologous DNA sequences, which, e.g., encode proteins for, e.g., the control of disease resistance or diagnostics of pathogen inducible or related gene

expression. The recombinant gene or vector containing the DNA sequence encoding an RNA or a protein of interest is introduced into the cells which in turn produce the RNA or protein of interest. For example, the chimeric promoter of the invention can be operatively linked to DNA sequences encoding Barnase for use in the production of localized cell death in plants upon pathogen attack.

On the other hand, said protein can be a scorable marker, e.g., luciferase, green fluorescent protein or ß-galactosidase. This embodiment is particularly useful for simple and rapid screening methods for compounds and substances described herein below capable of modulating pathogene specific or elicitor inducible gene expression. For example, transgenic plant cells can be cultured in the presence and absence of a candidate compound in order to determine whether the compound affects the expression of genes which are under the control of chimeric promoters of the invention, which can be measured, e.g., by monitoring the expression of the above-mentioned marker. It is also immediately evident to those skilled in the art that other marker genes may be employed as well, encoding, for example, a selectable marker which provides for the direct selection of compounds which induce or inhibit the expression of said marker.

The chimeric promoters of the invention may also be used in methods of antisense approaches. The antisense RNA may be a short (generally at least 10, preferably at least 14 nucleotides, and optionally up to 100 or more nucleotides) nucleotide sequence formulated to be complementary to a portion of a specific mRNA sequence and/or DNA sequence of the gene of interest. Standard methods relating to antisense technology have been described; see, e.g., Klann, Plant Physiol. 112 (1996), 1321-1330. Following transcription of the DNA sequence into antisense RNA, the antisense RNA binds to its target sequence within a cell, thereby inhibiting translation of the mRNA and down-regulating expression of the protein encoded by the mRNA.

Furthermore, appropriate ribozymes can be employed (see, e.g., EP-A1 0 291 533, EP-A1 0 321 201, EP-A2 0 360 257) which specifically cleave the (pre)-mRNA of a target gene. Selection of appropriate target sites and corresponding ribozymes can be done as described for example in Steinecke, Ribozymes, Methods in Cell Biology

50, Galbraith, eds Academic Press, Inc. (1995), 449-460. Further applications of the chimeric promoter are evident to the person skilled in the art and can be derived from the literature, e.g., Strittmatter and Wegener, Zeitschrift für Naturforschung 48c (1993), 673-688; Kahl, J. Microbiol. Biotechnol. 11 (1995), 449-460 and references cited therein.

Said transcription factor can for example be a master regulatory factor that controls the expression of a cascade of genes involved in pathogen defense of the plant (Grotewold, Plant Cell 10 (1998), 721-740; Rushton and Somssich, Curr. Opin. Plant Biol. 1 (1998), 311-315). Alternatively, it can be a hybrid transcription factor containing a DNA-binding domain (e.g. of GAL4 or of the bacteriophage 434) and an activator domain (e.g. of VP16 or of any functional plant activator domain), which, when expressed in transgenic plants containing an antisense target gene under the control of a synthethic promoter containing the appropriate cis-acting element recognizing the hybrid factor, leads to specific repression (knock-out) of the desired endogenous gene function (Wilde, Plant Mol. Biol. 24 (1994), 381-388; Guyer, Genetics 149 (1998), 633-639).

Suitable lipases comprise for example phospholipases, e.g., C or A_2 type phospholipases (Scherer, Plant Growth regulation 18 (1996), 125-133). Lipases are capable of releasing free fatty acids from membrane lipids, wherein these fatty acids can function as signal transducers by which general cellular defense reactions are elicited. The growing importance of free fatty acids in pathogen-defense is documented, e.g., in Scherer (1996), Roy (Plant Sci. 107 (1995), 17-25 and references cited therein) and Tavernier (Plant Sci. 104 (1995), 117-125).

Nucleases, i.e. RNases and DNases, may also be employed, of which Barnase is one candidate among others. The use of proteases in the context of this embodiment may apply to cytotoxic effects.

A signal amplification system may be constructed using polymerases. In a two-step model, an elicitor-induced polymerase, e.g., SP6-, T7- or T3-RNA polymerase, can transcribe a second recombinant gene which is controlled by a promoter to which the polymerase is highly specific. The second gene may encode for example a cytotoxic protein which is then expressed in an amplified way. A plant system based

on T7-RNA polymerase was described by McBride (Proc. Natl. Acad. Sci. USA 91 (1994), 7301-7305).

Cytotoxic proteins comprise, for example, plant RIPs (ribosome inactivating proteins; (Stripe, Bio/Technology 10 (1992), 405-412), defensins (Broekaert, Plant Physiol. 108 (1995), 1353-1358), Bt toxin, α-amylase inhibitor, T4-lysozyme, avirulence gene products, or enzymes such as glucose oxidase which generate reactive oxygen species (Shah, Trends Biotechnol. 13 (1995), 362-368; Shah, Curr. Opin. Biotech. 8 (1997), 208-214; Beachy, Curr. Opin. Biotech. 8 (1997), 215-220; Cornelissen, Plant Physiol. 101 (1993), 709-712; Estruch, Nucleic Acids Res. 22 (1994), 3983-3989).

It is in principle possible to modify the coding sequence in such a way that the protein is located in any desired compartment of the plant cell. These include the nucleus, endoplasmatic reticulum, the vacuole, the mitochondria, the plastids, the apoplast, the cytoplasm etc. Methods how to carry out this modifications and signal sequences ensuring localization in a desired compartment are well known to the person skilled in the art. (Görlich, Science 271 (1996), 1513-1518; Hicks, Plant Physiol. 107 (1995), 1055-1058; Rachubinski, Cell 83 (1995), 525-528; Schatz, Science 271 (1996), 1519-1526; Schnell, Cell 83 (1995), 521-524; Verner, Science 241 (1988), 1307-1313; Vitale, BioEssays 14 (1992), 151-160).

The present invention also relates to vectors, particularly plasmids, cosmids, viruses and bacteriophages used conventionally in genetic engineering that comprise a chimeric promoter or a recombinant gene of the invention. Preferably, said vector is a plant expression vector, preferably further comprising a selection marker for plants. For example of suitable selector markers, see supra. Methods which are well known to those skilled in the art can be used to construct recombinant vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the chimeric promoters and recombinant genes of the invention can be reconstituted into liposomes for delivery to target cells.

Advantageously, the above-described vectors of the invention comprise a selectable and/or scorable marker. Selectable marker genes useful for the selection of transformed plant cells, callus, plant tissue and plants are well known to those skilled in the art and comprise, for example, antimetabolite resistance as the basis of selection for dhfr, which confers resistance to methotrexate (Reiss, Plant Physiol. (Life Sci. Adv.) 13 (1994), 143-149); npt, which confers resistance to the aminoglycosides neomycin, kanamycin and paromycin (Herrera-Estrella, EMBO J. 2 (1983), 987-995) and hygro, which confers resistance to hygromycin (Marsh, Gene 32 (1984), 481-485). Additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); mannose-6-phosphate isomerase which allows cells to utilize mannose (WO 94/20627) and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.) or deaminase from Aspergillus terreus which confers resistance to Blasticidin S (Tamura, Biosci. Biotechnol. Biochem. 59 (1995), 2336-2338).

Useful scorable marker are also known to those skilled in the art and are commercially available. Advantageously, said marker is a gene encoding luciferase (Giacomin, Pl. Sci. 116 (1996), 59-72; Scikantha, J. Bact. 178 (1996), 121), green fluorescent protein (Gerdes, FEBS Lett. 389 (1996), 44-47) or ß-glucuronidase (Jefferson, EMBO J. 6 (1987), 3901-3907). This embodiment is particularly useful for simple and rapid screening of cells, tissues and plants containing a vector of the invention.

The present invention furthermore relates to host cells comprising a chimeric promoter, recombinant gene or a vector according to the invention wherein the chimeric promoter is foreign to the host cell.

By "foreign" it is meant that the chimeric promoter is either heterologous with respect to the host cell, this means derived from a cell or organism with a different genomic

background, or is homologous with respect to the host cell but located in a different genomic environment than the naturally occurring counterpart of said cis-acting element. This means that, if the cis-acting element is homologous with respect to the host cell, it is not located in its natural location in the genome of said host cell, in particular it is surrounded by different genes. The vector or recombinant gene according to the invention which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained in some form extrachromosomally. In this respect, it is also to be understood that the chimeric promoter or recombinant gene of the invention can be used to restore or create a mutant gene via homologous recombination (Paszkowski (ed.), Homologous Recombination and Gene Silencing in Plants. Kluwer Academic Publishers (1994)). The host cell can be any prokaryotic or eukaryotic cell, such as bacterial, insect, fungal, plant or animal cells. Preferred cells are plant cells.

In a further preferred embodiment, the present invention provides for a method for the production of transgenic plants, plant cells or plant tissue comprising the introduction of a chimeric promoter, recombinant gene or vector of the invention into the genome of said plant, plant cell or plant tissue. For the expression of the heterologous DNA sequence under the control of the chimeric promoter according to the invention in plant cells, further regulatory sequences such as poly A tail may be fused, preferably 3' to the heterologous DNA sequence, see also supra. Further possibilities might be to add Matrix Attachment Sites at the borders of the transgene to act as "delimiters" and insulate against methylation spread from nearby heterochromatic sequences.

Methods for the introduction of foreign genes into plants are also well known in the art. These include, for example, the transformation of plant cells or tissues with T-DNA using Agrobacterium tumefaciens or Agrobacterium rhizogenes, the fusion of protoplasts, direct gene transfer (see, e.g., EP-A 164 575), injection, electroporation, vacuum infiltration, biolistic methods like particle bombardment, pollen-mediated transformation, plant RNA virus-mediated transformation, liposome-mediated transformation, transformation using wounded or enzyme-degraded immature embryos, or wounded or enzyme-degraded embryogenic callus and other methods

known in the art. The vectors used in the method of the invention may contain further functional elements, for example "left border"- and "right border"-sequences of the T-DNA of Agrobacterium which allow stable integration into the plant genome. Furthermore, methods and vectors are known to the person skilled in the art which permit the generation of marker free transgenic plants, i.e. the selectable or scorable marker gene is lost at a certain stage of plant development or plant breeding. This can be achieved by, for example cotransformation (Lyznik, Plant Mol. Biol. 13 (1989), 151-161; Peng, Plant Mol. Biol. 27 (1995), 91-104) and/or by using systems which utilize enzymes capable of promoting homologous recombination in plants (see, e.g., WO97/08331; Bayley, Plant Mol. Biol. 18 (1992), 353-361); Lloyd, Mol. Gen. Genet. 242 (1994), 653-657; Maeser, Mol. Gen. Genet. 230 (1991), 170-176; Onouchi, Nucl. Acids Res. 19 (1991), 6373-6378). Methods for the preparation of appropriate vectors are described by, e.g., Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Suitable strains of Agrobacterium tumefaciens and vectors as well as transformation of Agrobacteria and appropriate growth and selection media are well known to those skilled in the art and are described in the prior art (GV3101 (pMK90RK), Koncz, Mol. Gen. Genet. 204 (1986), 383-396; C58C1 (pGV 3850kan), Deblaere, Nucl. Acid Res. 13 (1985), 4777; Bevan, Nucleic. Acid Res. 12(1984), 8711; Koncz, Proc. Natl. Acad. Sci. USA 86 (1989), 8467-8471; Koncz, Plant Mol. Biol. 20 (1992), 963-976; Koncz, Specialized vectors for gene tagging and expression studies. In: Plant Molecular Biology Manual Vol 2, Gelvin and Schilperoort (Eds.), Dordrecht, The Netherlands: Kluwer Academic Publ. (1994), 1-22; EP-A-120 516; Hoekema: The Binary Plant Vector System, Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V, Fraley, Crit. Rev. Plant. Sci., 4, 1-46; An, EMBO J. 4 (1985), 277-287). Although the use of Agrobacterium tumefaciens is preferred in the method of the invention, other Agrobacterium strains, such as Agrobacterium rhizogenes, may be used, for example if a phenotype conferred by said strain is desired.

Methods for the transformation using biolistic methods are well known to the person skilled in the art; see, e.g., Wan, Plant Physiol. 104 (1994), 37-48; Vasil, Bio/Technology 11 (1993), 1553-1558 and Christou (1996) Trends in Plant Science

1, 423-431. Microinjection can be performed as described in Potrykus and Spangenberg (eds.), Gene Transfer To Plants. Springer Verlag, Berlin, NY (1995).
The transformation of most dicotyledonous plants is possible with the methods described above. But also for the transformation of monocotyledonous plants several successful transformation techniques have been developed. These include the transformation using biolistic methods as, e.g., described above as well as protoplast transformation, electroporation of partially permeabilized cells, introduction of DNA using glass fibers, etc.
The resulting transformed plant cell can then be used to regenerate a transformed plant in a manner known by a skilled person.

Alternatively, a plant cell can be used and modified such that said plant cell expresses an endogenous gene under the control of the chimeric promoter. The introduction of the chimeric promoter of the invention which does not naturally control the expression of a given gene or genomic sequences using, e.g., gene targeting vectors can be done according to standard methods, see supra and, e.g., Hayashi, Science 258 (1992), 1350-1353; Fritze and Walden, Gene activation by T-DNA tagging. In *Methods in Molecular* biology 44 (Gartland, K.M.A. and Davey, M.R., eds). Totowa: Human Press (1995), 281-294) or transposon tagging (Chandlee, Physiologia Plantarum 78 (1990), 105-115).

In general, the plants which can be modified according to the invention can be derived from any desired plant species. They can be monocotyledonous plants or dicotyledonous plants, preferably they belong to plant species of interest in agriculture, wood culture or horticulture interest, such as crop plants (e.g. maize, rice, barley, wheat, rye, oats etc.), potatoes, oil producing plants (e.g. oilseed rape, sunflower, pea nut, soy bean, etc.), cotton, sugar beet, sugar cane, leguminous plants (e.g. beans, peas etc.), wood producing plants, preferably trees, etc.

Thus, the present invention relates also to transgenic plant cells comprising, preferably stably integrated into the genome, a chimeric promoter, a recombinant

gene or vector according to the invention or obtainable by the above-described method.

Furthermore, the present invention also relates to transgenic plants and plant tissue comprising the above-described transgenic plant cells or obtainable by the above-described method. These plants may show, for example, increased disease resistance.

In a preferred embodiment of the invention, the transgenic plant upon the presence of the chimeric promoter or the recombinant gene of the invention attained resistance or improved resistance against a pathogen the corresponding wild-type plant was susceptible to.

The term "resistance" covers the range of protection from a delay to complete inhibition of disease development. Examples for pathogens of importance comprise *Phytophthora infestans*, the causal agent of potato late blight disease, *Phytophthora sojae*, root rot pathogen of soybean, *Peronospora parasitica* (downy mildew), *Magnaporthe grisea*, causal agent of rice blast disease, *Erysiphe* spp (powdery mildew), *Pseudomonas syringae* (agent of bacterial blight), *Erwinia amylovora* (fire blight disease), *Erwinia carotovora* (soft rot), *Botrytis cinerea* (downy mildew of grape), *Rhizoctonia solani* and *Pythium debaryanum* (agents of seedling blight or damping off disease).

In yet another aspect the invention also relates to harvestable parts and to propagation material of the transgenic plants according to the invention which contain transgenic plant cells described above. Harvestable parts can be in principle any useful part of a plant, for example, leaves, stems, fruit, seeds, roots etc. Propagation material includes, for example, seeds, fruits, cuttings, seedlings, tubers, rootstocks etc.

As discussed above, novel cis-acting elements have been identified in accordance with the present invention that are capable of conferring elicitor inducible or

pathogen specific gene expression in plant cells and plants. Therefore, the present invention also relates to cis-acting elements as defined above or multimeric forms of any one of those as discussed hereinbefore.

Due to the tight regulation of the chimeric promoters of the invention it is evident that they are particularly suited for the identification of compounds that either specifically interact with these cis-acting elements or that act upstream of the signal transduction pathway that leads to activation of genes the cis-acting elements were derived from.

Thus, the present invention further relates to a method for the identification of an activator or inhibitor of genes specifically expressed in plants upon pathogen infection comprising the steps of:

(a) providing a plant, plant cell, or plant tissue comprising a recombinant DNA molecule comprising a readout system operatively linked to the chimeric promoter of the invention;

(b) culturing said plant cell or tissue or maintaining said plant in the presence of a compound or a sample comprising a plurality of compounds under conditions which permit expression of said readout system;

(c) identifying or verifying a sample and compound, respectively, which leads to suppression or activation and/or enhancement of expression of said readout system in said plant, plant cell, or plant tissue.

For the identification of inhibitors, it is advantageous to include an elicitor or an other activator known to be capable of inducing the activity of promoters that contain the cis-acting elements of the chimeric promoters of the invention in step (b) of the above-described method, and to determine whether the compound to be screened suppresses the induction of the readout system by said elicitor or activator.

The term "read out system" in context with the present invention means a DNA sequence which upon transcription and/or expression in a cell, tissue or organism provides for a scorable and/or selectable phenotype. Such read out systems are well known to those skilled in the art and comprise, for example, recombinant genes and marker genes as described above and in the appended examples.

The term "plurality of compounds" in a method of the invention is to be understood as a plurality of substances which may or may not be identical.

Said compound or plurality of compounds may be comprised in, for example, samples of inorganic or organic molecules or, e.g., cell extracts from, e.g., plants, animals or microorganisms. Furthermore, said compound(s) may be known in the art but hitherto not known to be capable of suppressing or activating pathogen related genes. Suitable set ups for the method of the invention are known to the person skilled in the art. The plurality of compounds may be, e.g., added to the cell or tissue culture medium or soil, injected into the cell or sprayed onto the plant.

If a sample containing a compound or a plurality of compounds is identified in the method of the invention, then it is either possible to isolate the compound from the original sample identified as containing the compound capable of suppressing or activating the chimeric promoter of the invention, or one can further subdivide the original sample, for example, if it consists of a plurality of different compounds, so as to reduce the number of different substances per sample and repeat the method with the subdivisions of the original sample. Depending on the complexity of the samples, the steps described above can be performed several times, preferably until the sample identified according to the method of the invention only comprises a limited number of or only one substance(s). Preferably said sample comprises substances of similar chemical and/or physical properties, and most preferably said substances are identical. Preferably, the compound identified according to the above described method or its analog or derivative is further formulated in a form suitable for the application in plant breeding or plant cell and tissue culture. For example, it can be combined with a agriculturally acceptable carrier known in the art.

The compounds which can be tested and identified according to a method of the invention may be expression libraries, e.g., cDNA expression libraries, peptides, proteins, nucleic acids, antibodies, small organic compounds, hormones, peptidomimetics, PNAs or the like (Milner, Nature Medicine 1 (1995), 879-880; Hupp, Cell 83 (1995), 237-245; Gibbs, Cell 79 (1994), 193-198 and references cited supra). Furthermore, genes encoding a putative regulator of genes controlled by the cis-acting elements of the invention and/or which excert their effects up- or

downstream from such genes may be identified using, for example, insertion mutagenesis using, for example, gene targeting vectors known in the art (see, e.g., Hayashi, Science 258 (1992), 1350-1353; Fritze and Walden, Gene activation by T-DNA tagging. In *Methods in Molecular* biology 44 (Gartland, K.M.A. and Davey, M.R., eds). Totowa: Human Press (1995), 281-294) or transposon tagging (Chandlee, Physiologia Plantarum 78 (1990), 105-115).
Said compounds can also be functional derivatives or analogues of known inhibitors or activators. Methods for the preparation of chemical derivatives and analogues are well known to those skilled in the art and are described in, for example, Beilstein, Handbook of Organic Chemistry, Springer edition New York Inc., 175 Fifth Avenue, New York, N.Y. 10010 U.S.A. and Organic Synthesis, Wiley, New York, USA. Furthermore, said derivatives and analogues can be tested for their effects according to methods known in the art or as described in the appended examples. Furthermore, peptidomimetics and/or computer aided design of appropriate derivatives and analogues can be used. The cell or tissue that may be employed in the method of the invention preferably is a plant cell, plant tissue or plant of the invention described in the embodiments hereinbefore.

In an additional embodiment, the characteristics of a given compound may be compared to that of a cell contacted with a compound which is either known to be capable or incapable of suppressing or activating the chimeric promoter of the invention or the promoter the cis-acting element of the chimeric promoter is derived from.

The inhibitor or activator identified by the above-described method may prove useful as a plant protective agent or herbicide or pesticide. Thus, in a further embodiment the invention relates to a compound obtained or identified according to the method of the invention said compound being an activator or an inhibitor of genes specifically induced upon pathogen infection.

Furthermore, identification of *trans*-acting factors which interact with the cis-acting elements of the invention can form the basis for the development of novel agents for

modulating conditions associated with plant diseases. Identification of *trans*-acting factors is carried out using standard methods in the art (see, e.g., Sambrook, supra, and Ausubel, supra). To determine whether a protein binds to the cis-acting elements of the invention standard DNA footprinting and/or native gel-shift analyses can be carried out. In order to identify the *trans*-acting factor which binds to the cis-acting elements of the invention, these elements can be used as an affinity reagent in standard protein purification methods, or as a probe for screening an expression library. Once the *trans*-acting factor is identified, modulation of its binding to the cis-acting elements of the invention can be pursued, beginning with, for example, screening for inhibitors of *trans*-acting factor binding.

Activation or repression of genes involved in plant defense reactions could then be achieved in plants by applying of the *trans*-acting factor (or its inhibitor) or the gene encoding it, e.g. in a vector for transgenic plants. In addition, if the active form of the *trans*-acting factor is a dimer, dominant-negative mutants of the *trans*-acting factor could be made in order to inhibit its activity. Furthermore, upon identification of the *trans*-acting factor, further components in the pathway leading to activation (e.g. signal transduction) or repression of pathogenesis related genes then can be identified. Modulation of the activities of these components can then be pursued, in order to develop additional agents and methods for modulating the response of plants upon pathogen attack in plants.

Accordingly, the present invention also relates to a plant protection composition comprising the compound identified and obtained by the above described methods. The plant protection composition can be prepared by employing the above-described method of the invention and synthesizing the compound identified as inhibitor or activator in an amount sufficient for use in agriculture. Thus, the present invention also relates to a method for the preparation of an agricultural plant protection composition comprising the above-described steps of the method of the invention and synthesizing the compound so identified or an analog or derivative thereof.

In the plant protection composition of the invention, the compound identified by the above-described method may be preferentially formulated by conventional means

commonly used for the application of, for example, herbicides and pesticides or agents capable of inducing systemic acquired resistance (SAR). For example, certain additives known to those skilled in the art stabilizers or substances which facilitate the uptake by the plant cell, plant tissue or plant may be used, for example, harpins, elicitins, salicylic acid (SA), benzol(1,2,3)thiadiazole-7-carbothioic acid (BTH), 2,6-dichloro isonicotinic acid (INA), jasmonic acid (JA), methyljasmonate.

In a further embodiment, the present invention relates to an antibody specifically recognizing the compound obtainable by the method of the invention or the cis-acting element described above. The antibodies of the invention can be used to identify and isolate other activators and inhibitors of genes in that are involved in plant defense. These antibodies can be monoclonal antibodies, polyclonal antibodies or synthetic antibodies as well as fragments of antibodies, such as Fab, Fv or scFv fragments etc. Monoclonal antibodies can be prepared, for example, by the techniques as originally described in Köhler and Milstein, Nature 256 (1975), 495, and Galfré, Meth. Enzymol. 73 (1981), 3, which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals. Furthermore, antibodies or fragments thereof to the aforementioned peptides can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988.

Furthermore, the present invention relates to a diagnostic composition comprising the chimeric promoter, the recombinant gene, the vector, the compound or the antibody of the invention, and optionally suitable means for detection. Said diagnostic compositions may be used for, e.g., methods for screening activators or inhibitors as described above.

In addition, the present invention relates to a kit comprising the chimeric promoter, the recombinant gene, the vector, the compound or the antibody of the invention. The kit of the invention may contain further ingredients such as selection markers and components for selective media suitable for the generation of transgenic plant cells, plant tissue or plants. Furthermore, the kit may include buffers and substrates

for reporter genes that may be present in the recombinant gene or vector of the invention. In addition, the kit of the invention may contain compounds such as elicitors, preferably fungal elicitors that can be used as standards for the expression assays. The kit of the invention may advantageously be used for carrying out the method of the invention and could be, inter alia, employed in a variety of applications referred to herein, e.g., in the diagnostic field or as research tool. The parts of the kit of the invention can be packaged individually in vials or in combination in containers or multicontainer units. Manufacture of the kit follows preferably standard procedures which are known to the person skilled in the art.

The kit or its ingredients according to the invention can be used in plant cell and plant tissue cultures, for example, for any of the above described methods for detecting inhibitors and activators of pathogenesis related genes. The kit of the invention and its ingredients are expected to be very useful in breeding new varieties of, for example, plants which display improved properties such as disease resistance.

It is also immediately evident to the person skilled in the art that the chimeric promoters, recombinant genes and vectors of the present invention can be employed to produce transgenic plants with a desired trait (see for review TIPTEC Plant Product & Crop Biotechnology 13 (1995), 312-397) comprising (i) insect resistance (Vaek, Plant Cell 5 (1987), 159-169), (ii) virus resistance (Powell, Science 232 (1986), 738-743; Pappu, World Journal of Microbiology & Biotechnology 11 (1995), 426-437; Lawson, Phytopathology 86 (1996), 56 suppl.), (iii) resistance to bacteria, insects and fungi (Duering, Molecular Breeding 2 (1996), 297-305; Strittmatter, Bio/Technology 13 (1995), 1085-1089; Estruch, Nature Biotechnology 15 (1997), 137-141), (iv) inducing and maintaining male and/or female sterility (EP-A1 0 412 006; EP-A1 0 223 399; WO93/25695) or may be used as highly inducible production systems of heterologous proteins or biopolymers in plants analogous to inducible systems in bacteria.

The present invention for the first time demonstrates that a number of cis-acting elements that are responsible for inducibility of pathogenesis-related genes can be used either alone or in combination with themselves or with other cis-acting

elements to construct chimeric promoters that are capable of mediating highly inducible gene expression in plant cells upon elicitor treatment. It is therefore evident that cis-acting elements derived, e.g., from pathogen-related promoters other than those specifically described above can be used in accordance with the present invention, for example, chitinase promoters; see, e.g., Kellmann, Plant. Mol. Biol. 30 (1996), 351-358. Appropriate promoters that provide a source for such cis-acting elements can be used and obtained from any plant species, for example, maize, potato, sorghum, millet, coix, barley, wheat and rice. Such promoters are characterized by their inducibility upon pathogen infection.

For example, using cDNA of proteins that are specifically expressed in plants upon pathogen attack as probes, a genomic library consisting of plant genomic DNA cloned into phage or bacterial vectors can be screened by a person skilled in the art. Such a library consists, e.g., of genomic DNA prepared from plant leaf tissue, fractionized in fragments ranging from 5 kb to 50 kb, cloned into the lambda vetors such as Lambda EMBL3 or 4, Lambda ZAP, Lambda DASH or Lambda GEM. Phages hybridizing with the probes can be purified. From the purified phages DNA can be extracted and sequenced. Having isolated the genomic sequences corresponding to the genes encoding the PR proteins, it is possible to fuse heterologous DNA sequences to these promoters or their regulatory sequences via transcriptional or translational fusions according to methods well known to the person skilled in the art. In order to identify the regulatory sequences and specific elements of the these genes, 5'-upstream genomic fragments can be cloned in front of marker genes such as luc, gfp or the GUS coding region and the resulting chimeric genes can be introduced by means of Agrobacterium tumefaciens mediated gene transfer into plants or transfected into plant cells or plant tissue for transient expression. The expression pattern observed in the transgenic plants or transfected plant cells containing the marker gene under the control of the isolated regulatory sequences reveal the boundaries of the promoter and its cis-acting elements. The isolation of cis-acting elements having the above defined properties can be done by conventional techniques known in the art, for example, by using DNAseI footprinting and loss-and gain-of-function experiments. It is then possible to

isolate the corresponding promoter region by conventional techniques and test it for its expression pattern. For this purpose, it is, for instance, possible to fuse the putative cis-acting element with a minimal promoter to a reporter gene, such as GUS, luciferase or green fluorescent protein (GFP) and assess the expression of the reporter gene in transient expression assays or transgenic plants; see also the appended examples.

Thus, the present invention relates to the use of a cis-acting element sufficient to direct elicitor-specific expression and in particular to the use of the chimeric promoter, the recombinant gene, the vector, the cis-acting element and/or the compound of the present invention for the production of pathogen resistant plants or for identifying and/or producing compounds capable of conferring induced resistance to a pathogen in a plant.

In a still further embodiment, the present invention relates to a method of rendering a gene responsive to pathogens comprising inserting at least one cis-acting element sufficient to direct elicitor-specific expression into the promoter of said gene. As is evident to the person skilled in the art a promoter that displays the capabilities of the chimeric promoter of the invention can also be obtained by introducing the cis-acting element as defined above into a promoter of a gene, preferably in close proximity to the transcription initiation site of the gene.

In another embodiment, the present invention relates to a method for preparing a promoter capable of mediating local gene expression in plants upon pathogen infection comprising operably linking a cis-acting element sufficient to direct elicitor-specific expression to a transcription initiation sequence of a promoter. Preferably, said cis-acting element to be inserted in the above-described methods is a cis-acting element of the present invention or as defined in the foregoing embodiments or a multimeric form thereof as defined hereinabove. As mentioned before, the elicitor responsive cis-acting elements are preferably responsive to fungal elicitor.

In a preferred embodiment of the invention, the above-described methods further comprising deleting non-specific cis-acting elements in the promoter. Introduction of the cis-acting element of the invention into a given promoter per se may not be sufficient to direct the promoter to exclusively mediate local gene expression in plants upon pathogen infection. In this case, preexisting elements that may be responsive, for example, to light, hormones, low temperatures, drought or salt stress may be deleted.

The above described methods give rise to novel chimeric promoters that are at least partially, preferably fully controlled by plant/pathogen interaction.

Accordingly, the present invention also relates to the promoter obtainable by a method as described above. Said promoter can then be employed for the embodiments described hereinabove.

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The present invention is further described by reference to the following non-limiting figures and examples.

The Figures show:

Figure 1 Restriction map of the plasmid ms23 (Sprenger, 1997)

Figure 2 Overview cartoon of the plasmid ms23. The Gus reporter gene and minimal -46 CaMV 35S promoter are shown, as are restriction sites found in the polylinker sequence situated 5´ to the minimal promoter. The distances (in base pairs) between the restriction sites are also shown.

Figure 3 Overview cartoon of the plasmid pGPTV. The Gus reporter gene and minimal -46 CaMV 35S promoter are shown as are the SpeI and XbaI sites used in making the constructs employed. The *nptII* selection marker is also indicated, as are the left and right T-DNA borders (L and R). The terminators (pApnos and pnos) and promoter driving the *nptII* gene (pAg7) are also shown.

Figure 4 Elicitor inducibility of chimeric promoters containing Box E17 and derivatives thereof. GTAC motifs in forward and reverse orientation are underlined. Deleted bases are depicted as Ø. The depicted fragments are located 12 bp upstream of the 35S minimal promoter. The monomers of the dimeric construct A109 are separated by a 6 bp restriction site.

Figure 5 Cut-out of the polylinker of the vector ms23. For measuring the influence of the distance to the 35S minimal promoter Box E17 or its dimer was inserted into eight different restriction sites.

Figure 6 Elicitor inducibility of Box E17 depending on the distance to the 35S minimal promoter. The induction upon elicitor treatment is given for the constructs as illustrated in Figure 5. ms23 represents the vector only containing the minimal promoter as negative control.

Figure 7 Expression characteristics of transgenic plants transformed with reporter gene constructs comprising chimeric promoters with tetramers of some cis-elements of the present invention. For comparison the GCC-Box element is included (see Example 1). The background expression levels are quantified as being low (barely detectable background expression), medium (visible background expression but induction by pathogens is clearly visible over the background) or very high (extremely high expression such that induction by pathogens is difficult to detect). A minus indicates no detectable expression, a plus indicates inducible expression and "nt" not tested.

The Examples illustrate the invention:

Experimental setup

1. Recombinant DNA techniques

Unless stated otherwise in the examples, all recombinant DNA techniques are performed according to protocols as described in Sambrook (1989), Molecular Cloning : A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY or in Volumes 1 and 2 of Ausubel (1994), Current Protocols in Molecular Biology, Current Protocols. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfase (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications (UK).

2. Transient expression vector

All constructs, unless a different protocol is given in the examples, were cloned between the SpeI and XbaI sites in pbt10-GUS (ms23) (Sprenger, 1997). At the 3' end of each construct is an intact XbaI site (6 bp) followed immediately by a minimal CaMV 35S promoter (-46 to +8). The 3' end of all inserts are therefore 28 bp upstream of the CaMV TATA Box and 52 bp upstream of the start of transcription. Multiple copies of the elements are separated by 6 base pairs (TCTAGT) created by the ligation of a SpeI sticky end with a XbaI sticky end. The sequence of ms23 (SEQ

ID NO: 17) as a restriction map and an overview cartoon are provided (Figures 1 and 2).

3. Transgenic plant vector

The vector employed was pGPTV-GUS-kan (Becker, Plant Mol. Biol. 20 (1992), 1195-1197). The polylinker, minimal CaMV 35S promoter and GUS reporter gene are identical to ms23. All spacings and orders of *cis*-elements within the constructs are therefore identical to those in the corresponding transient expression constructs in ms23. A cartoon of pGPTV is provided (Figure 3).

4. Transient transfection and expression assays

The transient transfection and expression assays were essentially carried out as described in Dangl, EMBO J. 6 (1987), 2551-2556; Schulze-Lefert, EMBO J. 8 (1989), 651-656; van de Löcht, EMBO J. 9 (1990), 2945-2950. Briefly, five day old subcultured parsley cells are used for the isolation of protoplasts. Protoplasting is achieved by overnight incubation of the cells in 0.24 M $CaCl_2$ containing 0.25% (w/v) cellulase and 0.05% (w/v) macerozyme at 24° C. Protoplasts are collected by centrifugation (7 min., 100 *g*), washed with 0.24 M $CaCl_2$, and then floated in B5 medium (GIBCO/BRL) containing 0.4 M sucrose and 1 mg/ml 2,4-dichlorophenoxyacetic acid. Protoplasts floating after centrifugation (5 min, 100 *g*) were harvested, counted and adjusted to 2×10^6/ml.

Supercoiled or linearized plasmid DNA (5 -20 µg) containing the chimeric promoter-reporter(GUS) construct was transferred into the protoplasts using the polyethylene glycol (PEG) method (Krens, Nature 296 (1982), 72-74). Each transformation assay was split and placed into two 3 ml plates. The Pep25 (Nürnberger, Cell 78 (1994), 449-460) elicitor was added to one whereas the other served as a control. Both samples were harvested after 8 hours, frozen in liquid nitrogen, crude protein extracts prepared and GUS activity assayed (Jefferson, Plant Mol. Biol. Rep. 5 (1987), 387-405). Bradford assays (Bio-Rad) were used for protein determination. The expression data are given as mean fold induction values ± standard deviation (SD) and mean GUS activity (pmol/min/mg) from six independent transient transfection assays treated with or without Pep25 elicitors.

5. Generation of transgenic plants

Transgenic plants were generated according to the methods described in Bechtold, Mol. Biol. Genet. 316 (1993), 1194-1199; Grant, Science 269 (1995), 843-846 and Dangl, Science 269 (1995), 843-846. Briefly, the promoter elements were cloned in front of the reporter gene of the binary vector pGPTV-GUS-kan (Becker, Plant Mol. Biol. 20 (1992), 1195-1197) and the constructs introduced into the Agrobacterium strain GV3101 (pMP90; (Koncz and Schell, loc. cit.) containing the pMP90 helper plasmid. 500ml cultures were grown in YEB medium containing kanamycin (50µg/ml), rifampicin (100µg/ml) and gentamycin (25µg/ml). Cells were resuspended in infiltration medium (0.5 x Murashige-Skoog salts; 1 x B5 vitamins; 5.0% sucrose and 0.044 µM benzlaminopurine) and vacuum infiltrated into Arabidopsis plants by the method of Grant (1995). T1 seeds were surface-sterilised and transformants were selected on MS medium containing 50µg/ml kanamycin. Primary transformants were transfered to soil and tested for GUS expression during pathogenesis and biotic or abiotic stress.

Example 1: Box S is capable of mediating elicitor induced gene expression

Box S (CAGCCACCAAAGAGGACCCAGAAT; SEQ ID NO: 7) has been shown to be necessary for the elicitor-responsive expression of the parsley *eli 7* genes (Takamiya-Wik, Ph.D. thesis, University of Köln, Köln, Germany (1995)). Together with the results concerning Box N (see Example 4.3) for the first time the core sequence of this type of element has been defined which appears to be AGCCACCANA The element is not identical to any known elicitor-responsive element although it is very similar to a number of ethylene response elements that have the core sequence AGCCGCC (GCC Boxes) (Ohme-Takagi and Shinshi, The Plant Cell 7 (1995), 173-182). In the promoters investigated (*eli7*-1, *eli7*-2 and *Prp1*) there is always an A residue rather than a G. What difference this difference in sequence makes is at present unclear and it is not known whether Box S is responsive to ethylene. It has however been shown for the first time that the Box S elements with the sequence AGCCACC are elicitor-responsive elements. The

present data also show for the first time that GCC Boxes are also elicitor response elements as well as being ethylene response elements. Box S is a very strong elicitor-responsive element. A monomer of Box S gives 11-fold inducibility and a tetramer up to 560-fold inducibility. This clearly shows Box S to be an extremely promising element for biotechnological purposes.

The sequence of the monomer element used is: 5'-actagtCAGCCACCAAAGAGGACCCAGAATtctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters. Constructs containing 1, 2, 4 and 8 copies of Box S were constructed and subjected to a transient expression assay as described above. The results were as follows:

	minus elicitor	Plus elicitor	Fold induction
1 x S	168	2058	12
2 x S	118	10781	91
4 x S	187	76904	441
8 x S	781	102211	130

These Box S constructs are novel and have high inducibilty. Four copies of Box S appears to be the best with a very low background value (187) a high induced level (76904) and a very high fold induction (441 x, the highest of any of the constructs tested).

Example 2: Box D is capable of mediating elicitor inducible gene expression

Box D (TACAATTCAAACATTGTTCAAACAAGGAACC; SEQ ID NO: 11) is present in the parsley PR2 promoter and has never before been reported to be a *cis*-acting element. Box D was identified by DNaseI footprinting, by loss of function experiments in the context of the PR2 promoter and by gain-of-function experiments with monomers and multimers. Box D is a very strong elicitor-responsive element, a tetramer directing 10-fold elicitor-inducibility combined with a very high level of expression, whilst a dimer is less strong but gives 15-20-fold inducibility. This clearly shows Box D to be a promising element for biotechnological purposes.

The sequence of the element used is: 5'-actagtTACAATTCAAACATTGTTCAAACAAGGAACCtctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters. Constructs containing 1, 2 and 4 copies of Box D were constructed and subjected to the transient expression assay described above. The results were shown below.

	minus elicitor	Plus elicitor	Fold induction
1 x D	346	4002	11
2 x D	1562	31331	20
4 x D	5519	61552	11

These Box D constructs are novel. Two copies of Box D may be the best with a moderate background value (1562), a high induced level (31331) and a good fold induction (20 x).

Example 3: Box U provides for elicitor inducible gene expression

Box U (ATGAAGTTGAAATTCAATAG; SEQ ID NO: 13) is present in the parsley PR2 promoter and has never before been reported to be a *cis*-acting element. Box U has been defined by DNaseI footprinting, by loss of function experiments in the

context of the PR2 promoter and by gain-of-function experiments with monomers and multimers. Box U is a reasonably strong elicitor-responsive element, a tetramer directing 40-fold elicitor-inducibility.

The sequence of the element used is: 5'-actagtAGTTGAAATTCAATAAGTTGAAATTCAATAtctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters.

Constructs containing 2 copies of the above Box U sequence were constructed. The results of a transient expression assay are shown below. These therefore contain 4 copies of the Box U element (AGTTGAAATTCAATA; SEQ ID NO: 12). 1 or 2 copies of Box U are also active.

	minus elicitor	Plus elicitor	Fold induction
4 x U	100	3947	39

These Box U constructs are novel. Box U appears to be a moderately strong pathogen-responsive element with a good fold induction (about 40 x).

Example 4: Some W Boxes are capable of mediating elicitor inducible gene expression

The results obtained in accordance with the present invention clearly show that there are great differences between the different W Boxes that have been tested. Some are very strong (Box W2), some weak (Box W1), some are not active at all on their own (Box W3) and some are present as composite elements together with other *cis*-acting elements (Box N). The W Boxes also have differences outside of the core TGAC sequences:

Box W1: TTTGACC (SEQ ID NO: 1)

Box W2: TTCAGCC-N_7-TTGACC (SEQ ID NO: 3)

Box W3: TGAC-N_6-GTCA (SEQ ID NO: 5)

Box N: TTTGACC plus GCCACC (S Box) (SEQ ID NO: 8)

Box W_{Amy}: TTGACC within TGAC-N_6-GTCA pallindrome (SEQ ID NO: 6)

4.1 Box W1

Box W1 (CACACTTAATTTGACCGAGTAACATTCGCC; SEQ ID NO: 2) has previously been identified as a weak elicitor-responsive *cis*-element in the parsley PR1 promoters and a tetramer has been shown to be sufficient to direct elicitor-responsive expression in the parsley transient expression system (Rushton, 1996). Box W1 contains the W box sequence TTGACC and evidence suggests that these elements are bound by the WRKY class of transcription factors. As W boxes have also been found in the monocots Wild oat (Rushton, 1995) and maize (Raventos, 1995) and WRKY proteins have been found in an increasing number of plant species this suggests that the W box elements may be *cis*-acting elements in all plant species. Box W1 had never before been tested on its own for activity as a monomer or in combination with other elements and it was observed that a monomer directs elicitor-inducible expression (5-fold inducibility) and that Box W1 is also active in combination with other elements (see below).

The current results show Box W1 itself, however, to be a weak element. The sequence of the element used (the monomer) is: 5'-actagtCACACTTAATTTGACCGAGTAACATTCGCCtctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters. This construct is slightly different than the construct previously reported (Rushton, 1996) as the element is inserted into the SpeI/XbaI sites and not BamHI/BglII. Constructs containing 1, 2 and 4 copies of Box W1 were constructed and subjected to the transient expression assay. The results were as follows.

	minus elicitor	Plus elicitor	Fold induction
1 x W1	362	1495	4.1
2 x W1	299	2433	8.1
4 x W1	56	870	<15

The fold induction with 4 x W1 is similar to the previously reported values (Rushton, 1996). Comparison with values for other elements shows Box W1 to be a weak element.

4.2 Box W2

Box W2 (TTATTCAGCCATCAAAGTTGACCAATAAT; SEQ ID NO: 4) has previously been identified as a *cis*-acting element required for the elicitor responsive expression of parsley PR1 promoters in the transient expression system (Rushton, 1996). However, gain of function has been first demonstrated in accordance with the present invention. Box W2, like Box W1, contains a TTGACC element but the rest of the element is totally different and these other sequences play an important role, as a tetramer of Box W1 is a weak element with about 10-fold elicitor inducibility whereas Box W2 directs levels of expression up to 100 times higher than Box W1 with a 50-fold elicitor inducibility. It is shown for the first time that Box W2 alone, as a monomer or multimer, is a very strong elicitor-responsive element and that it is also active in combination with other elements.

The sequence of the element used (the monomer) is: 5'-actagtTTATTCAGCCATCAAAGTTGACCAATAATctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters. Constructs containing 1, 2, 4 and 8 copies of Box W2 were constructed and subjected to the transient expression assay. The following results were obtained.

	minus elicitor	Plus elicitor	Fold induction
1 x W2	770	8914	11
2 x W2	998	46651	46
4 x W2	2375	105685	44
8 x W2	7680	164454	21

W2 is the strongest elicitor-responsive *cis*-acting element that has been so far tested, eight copies of W2 giving GUS values of approximately 164,000.

4.3 Box N

Box N comes from the potato *gst1* gene (TTCTAGCCACCAGATTTGACCAAAC; SEQ ID NO: 9) and has never previously been defined. It contains both an S Box sequence (AGCCACCAGA) and a W Box sequence (TTGACC) within just 25 base pairs and as such represents a novel *cis*-element composed of two types of elicitor response element within a very small stretch of DNA. A tetramer of Box N gives at least 75-fold elicitor inducibility. This observation suggests three important conclusions; firstly that Box N may be extremely useful for biotechnological applications, secondly that the core Box S sequence is AGCCACCANA (SEQ ID NO: 14) and thirdly that Boxes S and W may represent a common theme in plant promoters that respond to pathogens as these elements are present in both parsley and potato. Box N alone is a strong elicitor-responsive element and extremely interesting, as it consists of an S Box (GCCACC) followed by a W Box (TTTGACC). The sequence of the element used (the monomer) is: 5'-actagtTTCTAGCCACCAGATTTGACCAAACtctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters. A construct with four copies of Box N was tested in transient expression assay. The results were as follows.

	minus elicitor	Plus elicitor	Fold induction
4 x N	1085	92980	85

Box N is a strong element and shows a very high fold inducibity. This novel combination and spacing of W and S Box elements may prove to be very useful for biotechnological purposes.

4.4 Box W_{Amy}

Box W_{Amy} comes from the wild oat *α-Amy2/A* and wheat *α-Amy2/54* genes where it has previously been published under the name Box 2 or O2S (see Rushton, Plant Mol. Biol. 29 (1995), 691-702). It is a *cis*-acting element required for the transcriptional activation of these genes during germination but has never previously been linked to a role in pathogenesis. Box W_{Amy} consists of two W Box elements: a hexamer 5'-TTGACC-3' embedded in a pallindromic 5'-TGAC-N_6-GTCA-3'. As it contains both types of sequences together it constitutes a new type of W Box and may be a "super W Box".

The sequence of the element used (the monomer) is: 5'-actagtGGATTGACTTGACCGTCATCGGCTtctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters. A construct containing 7 copies of Box W_{Amy} was constructed and to the transient expression assay. The result is shown below.

	minus elicitor	Plus elicitor	Fold induction
7 x W_{Amy}	168	43867	260

W_{Amy} is a strong elicitor-responsive *cis*-acting element and has the highest fold induction of any W Box that has been so far tested. This element could therefore be a particularly effective W Box and could aid the designing of synthetic W Boxes that are even more effective

Example 5: Synthetic promoters consisting of combinations of the above-described elements

Synthetic promoters composed of combinations of the above elicitor-responsive elements have never before been constructed or tested. All elements (Boxes W1, W2, S, U, D, N and W_{Amy}) are active in combination with each other; monomer, dimer and tetramer constructs being active. The furthest downstream element (nearest to the TATA Box) has the strongest effect on the synthetic promoter with further upstream elements having a much lesser effect. However the combination of two or more different types of *cis*-element may have a much more profound effect on expression *in planta*. In addition the insertion of a spacer region composed of anything between 100 base pairs and 1,000 base pairs appears to increase the contribution of the more upstream *cis*-elements. All of these synthetic promoters are good candidate promoters that may be rapidly and locally responsive to pathogen attack but also show negligible activity in uninfected tissues. These promoters may therefore allow the engineering of defence reactions that are closely related to natural defence mechanisms without appreciable activity in non-infected cells of the plant.

A large number of combinations have been tested. The results for some of these are detailed below. All of these combinations are novel and these constructs represent true synthetic promoters. The elements are inserted into the SpeI/XbaI sites, as with all of the constructs, and read from the 5' end to the 3' end i.e 4 x W2/ 4 x S is:
SpeI - W2- W2 - W2 - W2 - S- S -S -S- XbaI
Generally, the elements nearest to the TATA Box (i.e at the 3' end) have the greatest effect on both level of expression and fold induction. The effect of the upstream elements is often minimal and there is also an inhibitory effect probably due to steric hinderance when different elements are put close together; compare 4 x S/ 4 x W2 with (2 x S/ 2 x W2) x 2. The insertion of spacer regions between elements is therefore recommended to alleviate problems due to steric hinderance. The results of the transient expression assays are shown below.

	minus elicitor	Plus elicitor	Fold induction
1 x S/ 1 x W2	1732	85126	49
2 x S/ 2 x W2	1529	95872	62
4 x S/ 4 x W2	2654	64105	24
(2 x S/ 2 x W2) x 2	483	9832	20
4 x W2/ 4 x S	2753	205826	74
1 x W2/ 1 x S	146	2690	18
2 x S/ 2 x D	191	15541	81
4 x S/ 4 x D	9775	100265	10
1 x D/ 1 x S	32	1246	38
4 x D/ 4 x S	6795	204115	30
2 x W2/ 2 x D	1762	32462	18
4 x W2/ 4 x D	22042	92875	4.2
4 x D/ 4 x W2	18857	276456	14
1 x D/ 1 x W2	295	4369	14

Adding more copies of an element in a composite construct often increases the absolute level of expression (e.g 2 x W2/ 2 x D and 4 x W2/ 4 x D) but often lowers the fold induction. In some cases even the absolute level of expression decreases (e.g 2 x S/ 2 x W2 and 4 x S/ 4 x W2) and a comparison with (2 x S/ 2 x W2) x 2 suggests that this is due to steric hinderance as the number of copies of the elements is the same, it is just the order that is changed.

Example 6: Box E17 is capable of mediating elicitor induced gene expression

Box E17 (TCAATATGTCAATGGTCAACATTCAAC; SEQ ID NO: 15) was isolated from the promoter of the parsley Eli17 gene which is known to react to elicitor-treatment with transcript accumulation (Somssich, Plant Mol. Biol. 12 (1989), 227-234). Recently it has been shown that the Eli17 gene reacts very rapidly and transiently to elicitor-treatment and pathogen infection. This has never been previously described.

The sequence of the monomer element used is: 5'-actagtTCAATATGTCAATGGTCAACATTCAACtctaga-3' with the element in upper case letters and the SpeI/XbaI ends in lower case letters. Constructs containing 1 and 2 copies of Box E17 as well as a monomeric reverse complement of Box E17 were constructed (Figure 4, constructs B109, A109, and 18S102, respectively) and subjected to a transient expression assay as described above. As shown in Figure 4, the monomer has 5-fold inducibility and the dimer 50-fold. In comparison to the other cis-elements of the present invention moderate induction was achieved by Box E17. Likewise, a tetramer of Box E17 was subjected to transient assays (data not shown), which resulted in 5- to 20-fold induction following elicitor-treatment. However, this result cannot be compared to the induction values of the Box E17 constructs mentioned above because of diminished quality of the parsley protoplasts used. Presumably, the Box E17 tetramer mediates at least an induction as high-fold as the respective dimer.

Similar to cis-elements of Example 4 Box E17 contains two copies of the W-Box core motif TGAC, in reverse orientation (GTCA) as tandem repeat seperated by a 3 bp spacer. The importance of this core motif can be inferred from preliminary mutagenesis experiments (Figure 4, constructs C109, 17S102, and 15S102). A 1 bp deletion within the W-Box motif resulted in complete loss of function in contrast to deletions at two different sites having no effect to inducibility. This finding together with the results of Example 4 led to the definition of the core sequence derived from Box E17: GTCANNNGTCA (SEQ ID NO: 16). The element is not identical to any known elicitor-responsive element. However, it is contained in the M4 oligonucleotide GATTTGGTCAGAAGTCAGTCC, which is a mutated version of a sequence derived from the tobacco CHN50 promoter and which actively binds proteins of nuclear extracts of elicitor-treated tobacco cells (Fukuda; Plant Mol. Biol. 34 (1997), 81-87). But since no function with respect to promoter inducibility has been documented for M4, the element GTCANNNGTCA is for the first time described to mediate induction of gene expression upon elicitor-treatment and is not contained in any other known elicitor-responsive element.

Example 7: Chimeric promoters with varying distances of the Box E17 element to the minimal promoter are inducable.

In order to elucidate the optimal position of the Box E17 element within the chimeric promoter several constructs with varying distances to the 35S minimal promoter were tested (Figures 5 and 6). For this purpose Box E17 was inserted into different restriction sites of the ms23 polylinker. After digesting the vector and filling in the overhangs the cis-element was blunt ligated into the respective site as a monomer or as a dimer. The transient assays were conducted as described above. The results (Figure 6) indicate an optimal distance of Box E17 to the 5' end of the minimal promoter of 40 to 60 bp (corresponding to the restriction sites BamHI, ClaI, EcoRI). Still good induction was observed for the SapI site in 131 bp distance whereas considerably weaker response was obtained when Box E17 was inserted into the SalI site which is 5 bp upstream of the minimal promoter.

Example 8: Transgenic plants carrying chimeric promoters

Transformants were tested for the response of the synthetic promoters to pathogens. Cultures of the bacterium pseudomonas (strains *Rpt2* or *Rpm1*) were grown in King's-B Medium containing 30 µg/ml kanamycin and 100µg/ml rifampicin. The bacteria were resuspended in 10mM $MgCl_2$ at an OD_{600} of 0.2 and infiltrated into leaves via a syringe. Controls were performed using 10mM $MgCl_2$ alone. After 6 hours the leaves were removed from the plants and stained for GUS activity using X-Gluc. The expression pattern observed in the transgenic plants containing the GUS marker gene under the control of the chimeric promoter of the invention revealed expression in tissue infected by *Pseudomonas syringae* and in some cases also local expression in wounded tissues.

With regard to Box E17 a chimeric promoter comprising the dimer of this element (A109, Figure 5) and the 35S minimal promoter was used for transformation of Arabidopsis plants. Two to three weeks old seedlings and old leaves of the transformants were infiltrated with a 10µM aqueous solution of the bacterial elicitor

Flagellin 22 via a syringe (Felix, Plant Journal 18 (1999) 262-276; Gómez-Gómez, Plant Journal 18 (1999) 277-284) which led to clear GUS activation. High induction was also observed after infection by a fungal *(Peronospora parasitica)* and a bacterial pathogen *(Pseudomonas syringae)*.

Peronospora infections were carried out according to Dangl et al. (Genetic definition of loci involved in Arabidopsis-pathogen interactions. In: Methods in Arabidopsis Research (Koncz, Chua and Schell, eds.). Singapore: World Scientific Publishing Co. (1992), 393-418) or Koch (Plant Cell 2 (1990), 437-446).

On the other hand, mechanical stress induced for example by wounding did not activate the chimeric promoter. And surprisingly, no or only mere expression and activation of the reporter gene was observed in root which is the organ where the Eli17 gene is predominantly expressed in parsley. Thus, organ specificity appears not to be mediated by Box E17.

Furthermore, expression studies were performed which results are summarized in Figure 7. Seven different tetramers of cis-elements were assayed for their background expression in aerial parts and roots, respectively, and for their inducibility after wounding, senescence, incompatible and compatible *Peronospora* infection. Some important conclusions can be drawn from these experiments:

All of these chimeric promoters that are inducible by incompatible strains of *Peronospora parasitica* are also inducible by compatible strains. This is an important observation regarding the present invention as it shows that these constructs could be inducible by all potential pathogens and not just those for which there is already a functional defense system in operation in the plant.

Although many constructs show induced expression around infection sites, the expression characteristics are different with, for example, some W Boxes (e.g. W2) being expressed in an area around the infection site whereas others are expressed within the infection site itself. This is an unexpected finding as it shows that within a class of cis-acting elements (W Boxes or GCC/S Boxes) differences in sequence outside of the core sequence lead to differences in functionality.

All of the cis-acting elements of the present invention show inducible expression in a heterologous plant (*Arabidopsis*). As these elements come from parsley, potato and

wheat this clearly shows that these elements could be functional in all plants. This general functionality of such elements is an important new observation.

CLAIMS

1. A chimeric promoter capable of mediating local gene expression in plants upon pathogen infection comprising
   (i) at least one cis-acting element sufficient to direct elicitor-specific expression comprising the nucleotide sequence of any one of SEQ ID NOS: 3 to 16, and
   (ii) a minimal promoter.

2. The chimeric promoter of claim 1, further comprising a cis-acting element having the nucleotide sequence of SEQ ID NO: 1 or 2.

3. The chimeric promoter of claim 1 or 2, wherein said synthetic plant promoter comprises homo- and/or hetero-multimeric forms of said cis-acting element(s).

4. The chimeric promoter of any one of claims 1 to 3, wherein said multimeric form is a dimer or tetramer.

5. The chimeric promoter of any one of claims 1 to 4, wherein the minimal promoter is derived from the CaMV35S promoter, CHS promoter, PR1 promoter, or hcbt2 promoter.

6. The chimeric promoter of any one of claims 1 to 5, wherein the distance between said cis-acting element and said minimal promoter is 12 to 300 base pairs, more preferably 25 to 70 base pairs, and most preferably 40 to 60 base pairs.

7. The chimeric promoter of any one of claims 1 to 6, wherein a spacer region composed of 4 to 10 base pairs separates at least two of said cis-acting elements.

8. The chimeric promoter of any one of claims 3 to 7, wherein at least two of said multimeric forms are separated by a spacer of between about 50 to 1000 base pairs.

9. The chimeric promoter of any one of claims 1 to 8, wherein the induction of gene expression upon elicitor treatment or pathogen infection is at least 15-fold.

10. A recombinant gene comprising the chimeric promoter of any one of claims 1 to 9.

11. The recombinant gene of claim 10, wherein the chimeric promoter is operatively linked to a heterologous DNA sequence.

12. The recombinant gene of claim 10 or 11, wherein at least one of said cis-acting elements is located in the 5'- or 3-untranslated region or in an intron of the recombinant gene.

13. The recombinant gene of any one of claims 10 to 12, wherein said heterologous DNA sequence encodes a (poly)peptide, cytotoxic protein, antibody, antisense RNA, sense RNA, ribozyme, transcription factor, protease, nuclease, lipase, or polymerase

14. A vector comprising the chimeric promoter of any one of claims 1 to 9 or the recombinant gene of any one of claims 10 to 13.

15. A method for the production of transgenic plants, plant cells or plant tissue comprising the introduction of a chimeric promoter of any one of claims 1 to 9, a recombinant gene of any one of claims 10 to 13 or the vector of claim 14 into the genome of said plant, plant cell or plant tissue.

16. Plant cells comprising a chimeric promoter of any one of claims 1 to 9, the recombinant gene of any one of claims 10 to 13 or the vector of claim 14 or obtainable by the method of claim 15.

17. A transgenic plant or plant tissue comprising plant cells of claim 16.

18. The transgenic plant of claim 17, which upon the presence of the chimeric promoter or the recombinant gene attained resistance or improved resistance against a pathogen the corresponding wild-type plant was susceptible to.

19. Harvestable parts of a transgenic plant of claim 17 or 18 comprising plant cells of claim 16.

20. Propagation material of a transgenic plant of claim 17 or 18 comprising plant cells of claim 16.

21. A cis-acting element as defined in claim 1 or a multimeric form(s) of any one of those as defined in claim 3 or 4.

22. A method for the identification of an activator or inhibitor of genes specifically expressed in plants upon pathogen infection comprising the steps of:

(a) providing a plant, plant cell, or plant tissue comprising a recombinant DNA molecule comprising a readout system operatively linked to the chimeric promoter of any one of claims 1 to 9;

(b) culturing said plant cell or tissue or maintaining said plant in the presence of a compound or a sample comprising a plurality of compounds under conditions which permit expression of said readout system;

(c) identifying or verifying a sample and compound, respectively, which leads to suppression or activation and/or enhancement of expression of said readout system in said plant, plant cell, or plant tissue.

23. The method of claim 22 further comprising the step of

(d) subdividing the samples identified in step (c) and repeating steps (a) to (c) one or more times.

24. The method of claim 22 or 23 further comprising the step of

(e) identifying and/or isolating from the identified sample the compound responsible for said suppression or activation and/or enhancement of expression of said readout system in said plant, plant cell, or tissue.

25. The method of any one of claims 22 to 24, wherein

(a) said recombinant DNA molecule is a recombinant gene of any one of claims 10 to 13 or a vector of claim 14;

(b) said plant cell is a plant cell of claim 16;

(c) said plant tissue is a plant tissue of claim 17, or

(d) said plant is a plant of claim 17 or 18.

26. A method for preparing a plant elicitor comprising the steps of the method of any one of claims 22 to 25 and formulating the compound obtained or identified in step (c) or (e) in a form suitable for the application in agriculture or plant cell and tissue culture.

27. A compound obtained or identified by the method of any one of claims 22 to 26 which is an activator or inhibitor of gene expression and/or function in plants.

28. An antibody specifically recognizing the compound of claim 27 or the cis-acting element of claim 21.

29. A diagnostic composition comprising a chimeric promoter of any one of claims 1 to 9, the recombinant gene of any one of claims 10 to 13, the vector of claim 14, the compound of claim 27 or the antibody of claim 28, and optionally suitable means for detection.

30. A kit comprising a chimeric promoter of any one of claims 1 to 9, the recombinant gene of any one of claims 10 to 13, the vector of claim 14, the compound of claim 27 or the antibody of claim 28.

31. A plant protection composition comprising the compound of claim 27.

32. Use of a cis-acting element sufficient to direct elicitor-specific expression, a chimeric promoter of any one of claims 1 to 9, the recombinant gene of any one of claims 10 to 13, the vector of claim 14, the cis-acting element of claim 21 and/or the compound of claim 27 for the production of pathogen resistant plants.

33. Use of a cis-acting element sufficient to direct elicitor-specific expression, the chimeric promoter of any one of claims 1 to 9, a recombinant gene of any one of claims 10 to 13, a vector of claim 14, the plant cell of claim 16, the plant tissue of claim 17, or the plant of claim 17 or 18 for identifying and/or producing compounds capable of conferring induced resistance to a pathogen in a plant.

34. A method of rendering a gene responsive to pathogens comprising inserting at least one cis-acting element sufficient to direct elicitor-specific expression into the promoter of said gene.

35. A method for preparing a promoter capable of mediating local gene expression in plants upon pathogen infection comprising operably linking a cis-acting element sufficient to direct elicitor-specific expression to a transcription initiation sequence of a promoter.

36. The method of claim 34 or 35, wherein said cis-acting element is a cis-acting element as defined in claim 1 or 2 or a multimeric form thereof as defined in any one of claims 3 to 8.

37. The method of any one of claims 34 to 36, further comprising deleting non-specific cis-acting elements in the promoter.

38. The promoter obtainable by the method of any one of claims 34 to 37.

39. Use of the compound of claim 27 as plant protective agent or herbicide.

SEQUENCE LISTING

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Fig. 1

Restriction map of plasmid ms23

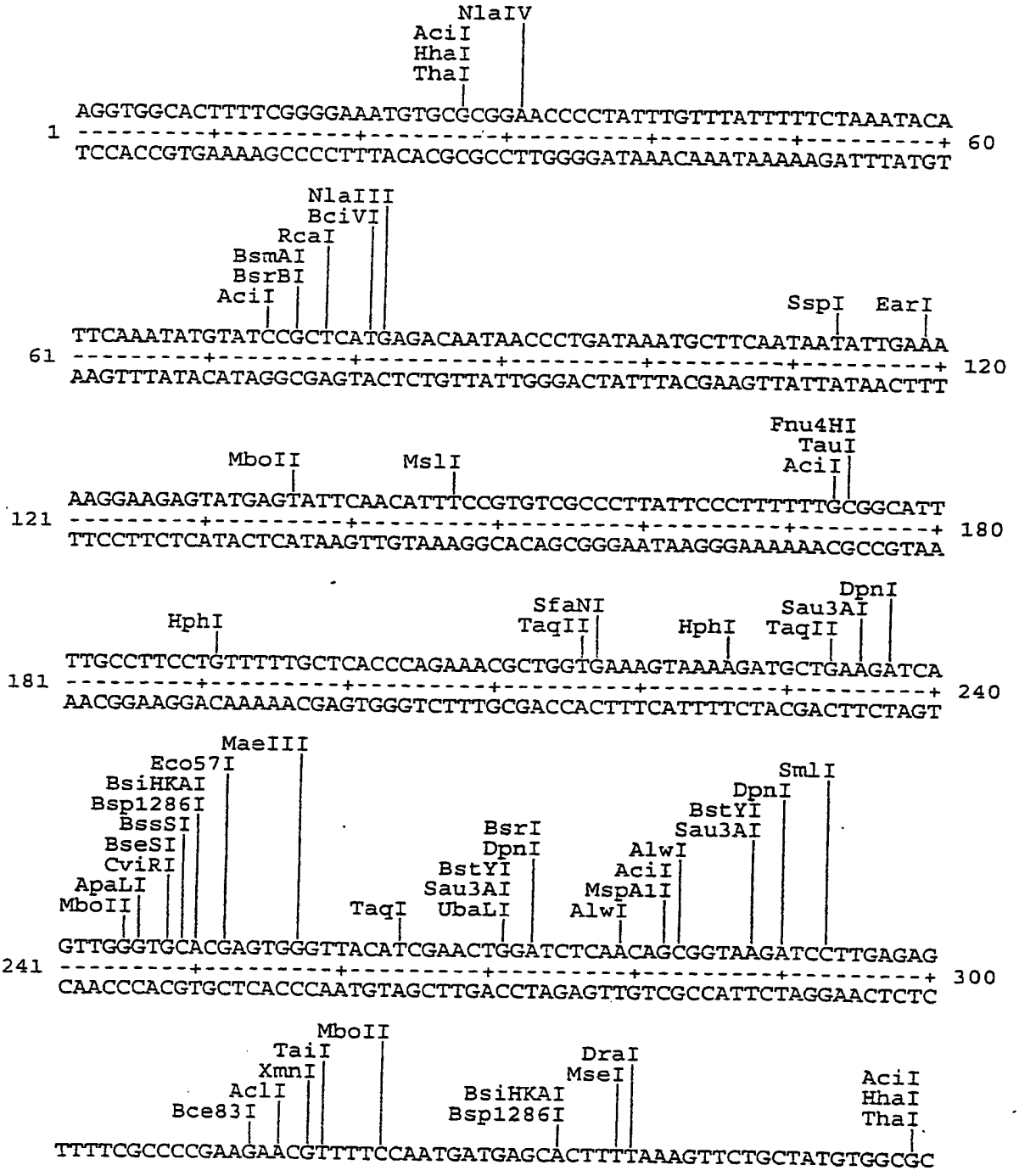

Fig. 1(cont.)

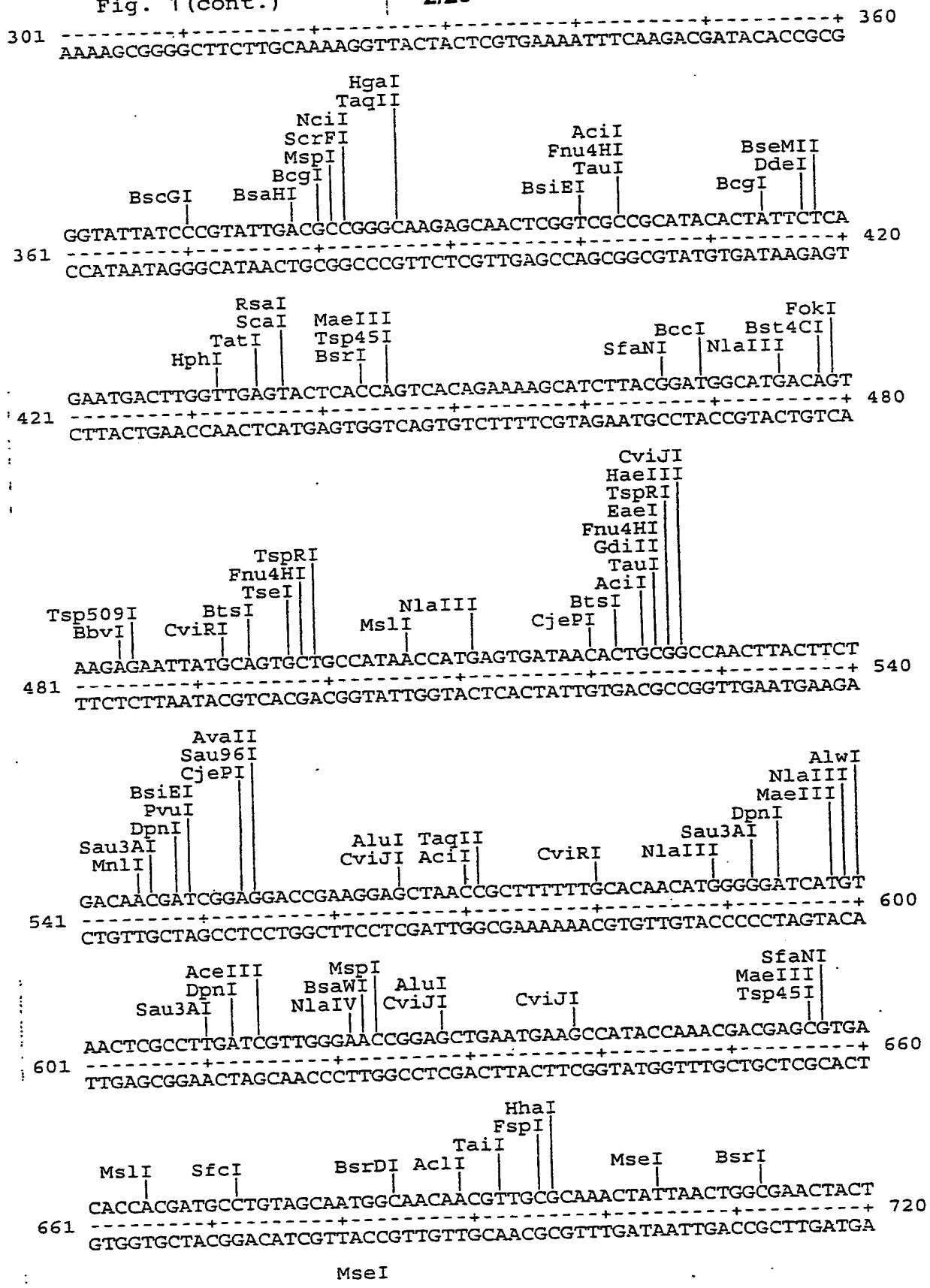
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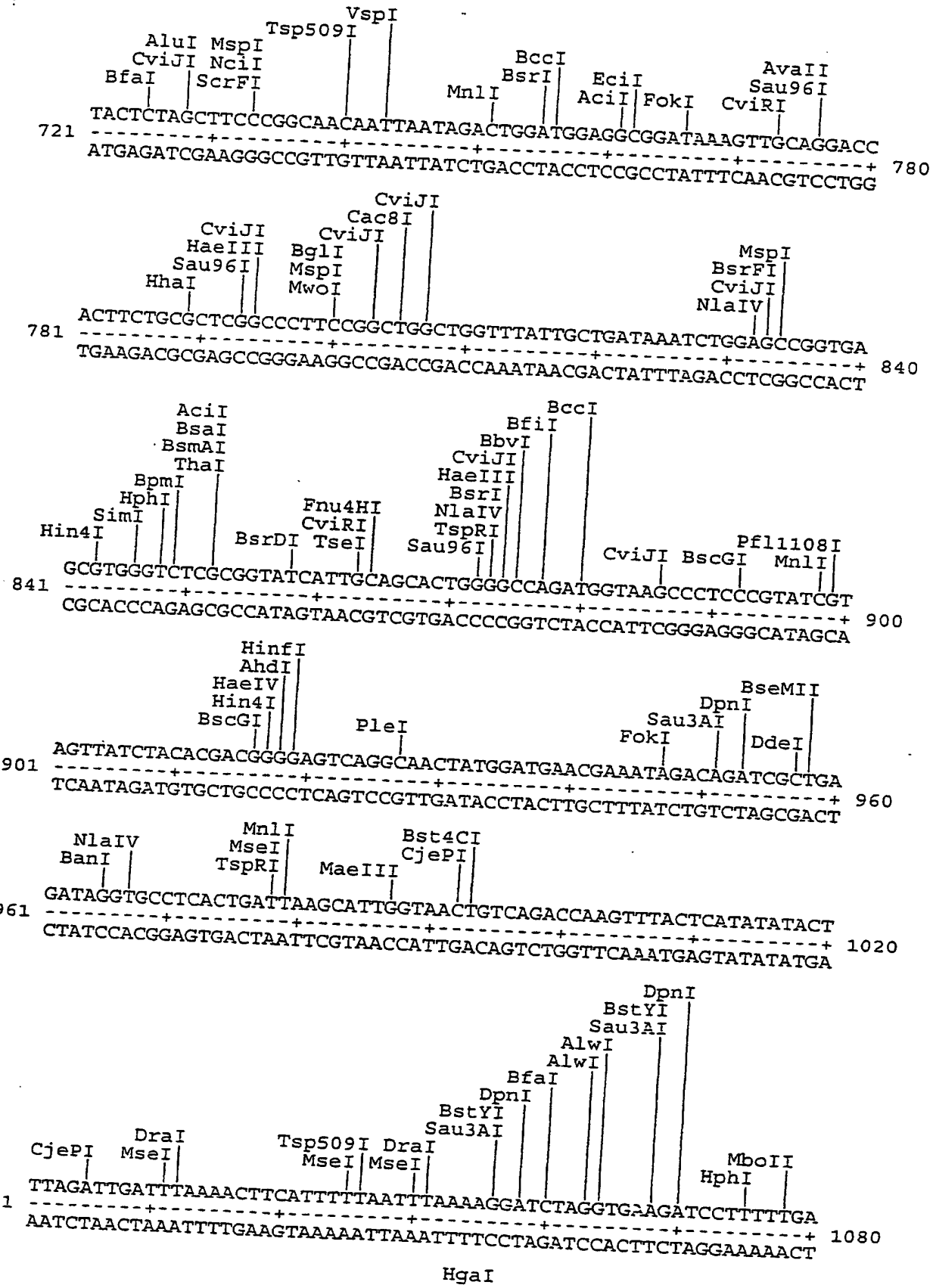
Fig.1(cont.) 3/20



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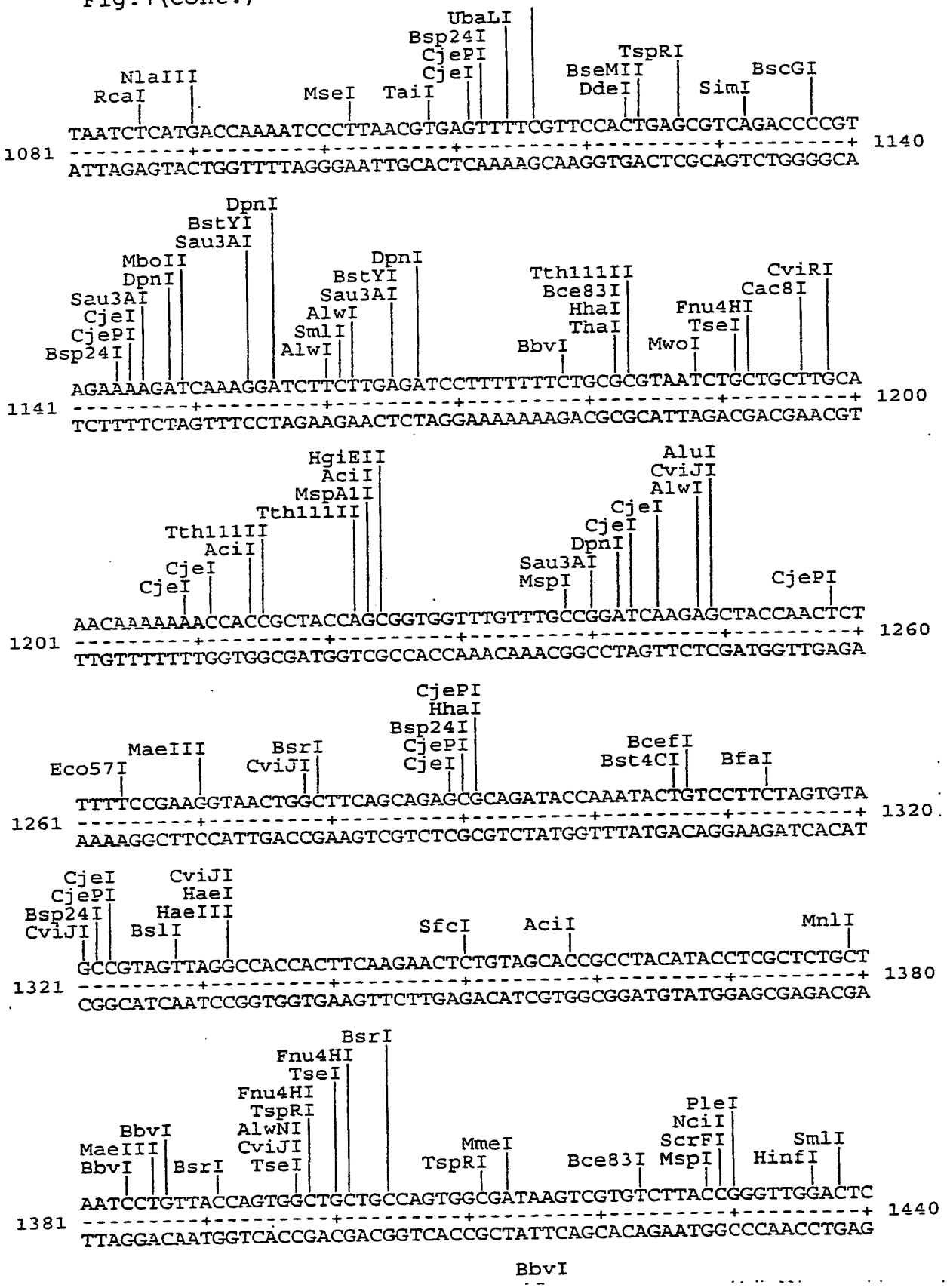
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Fig.1(cont.)

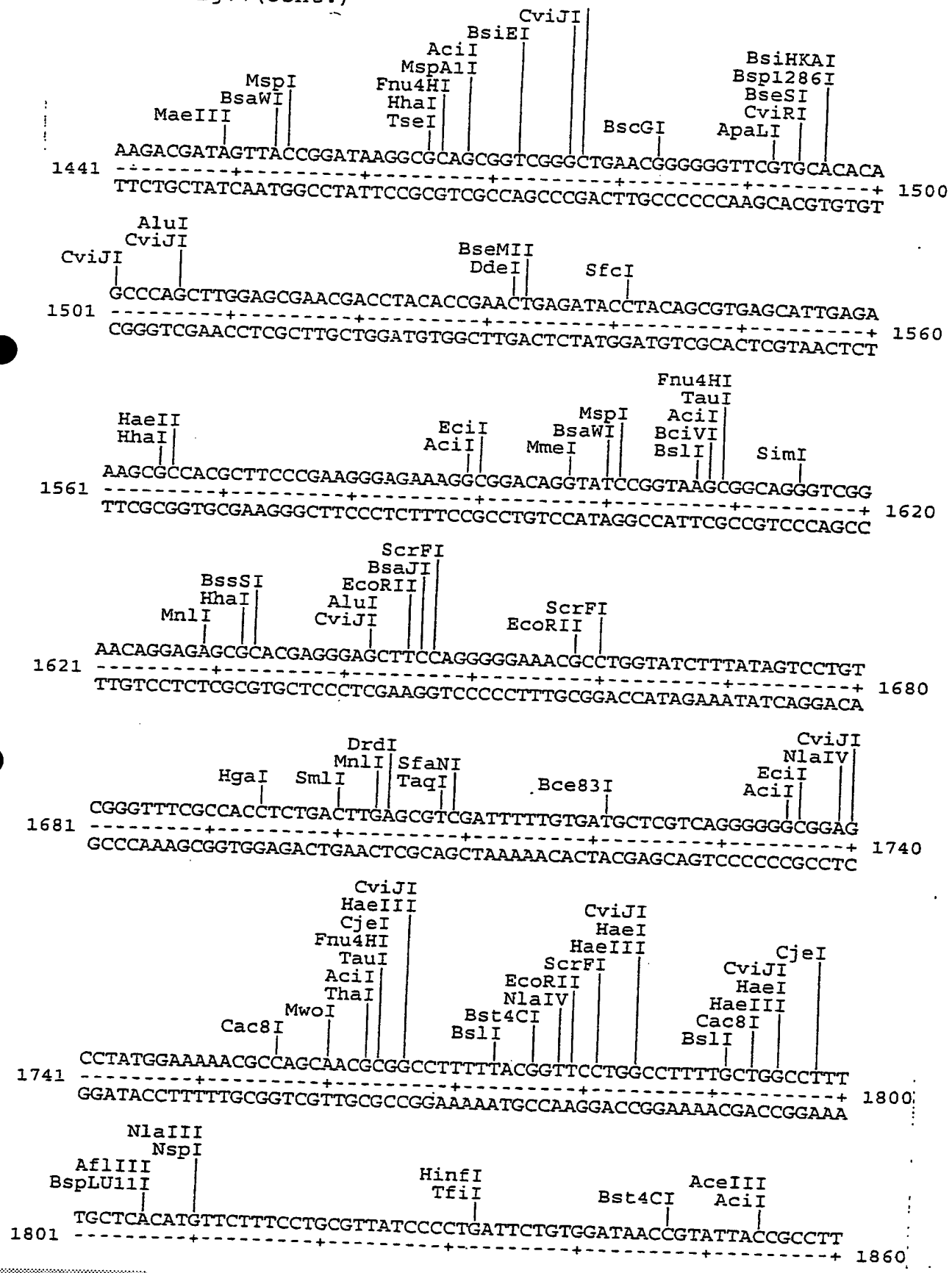
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Fig.1(cont.)

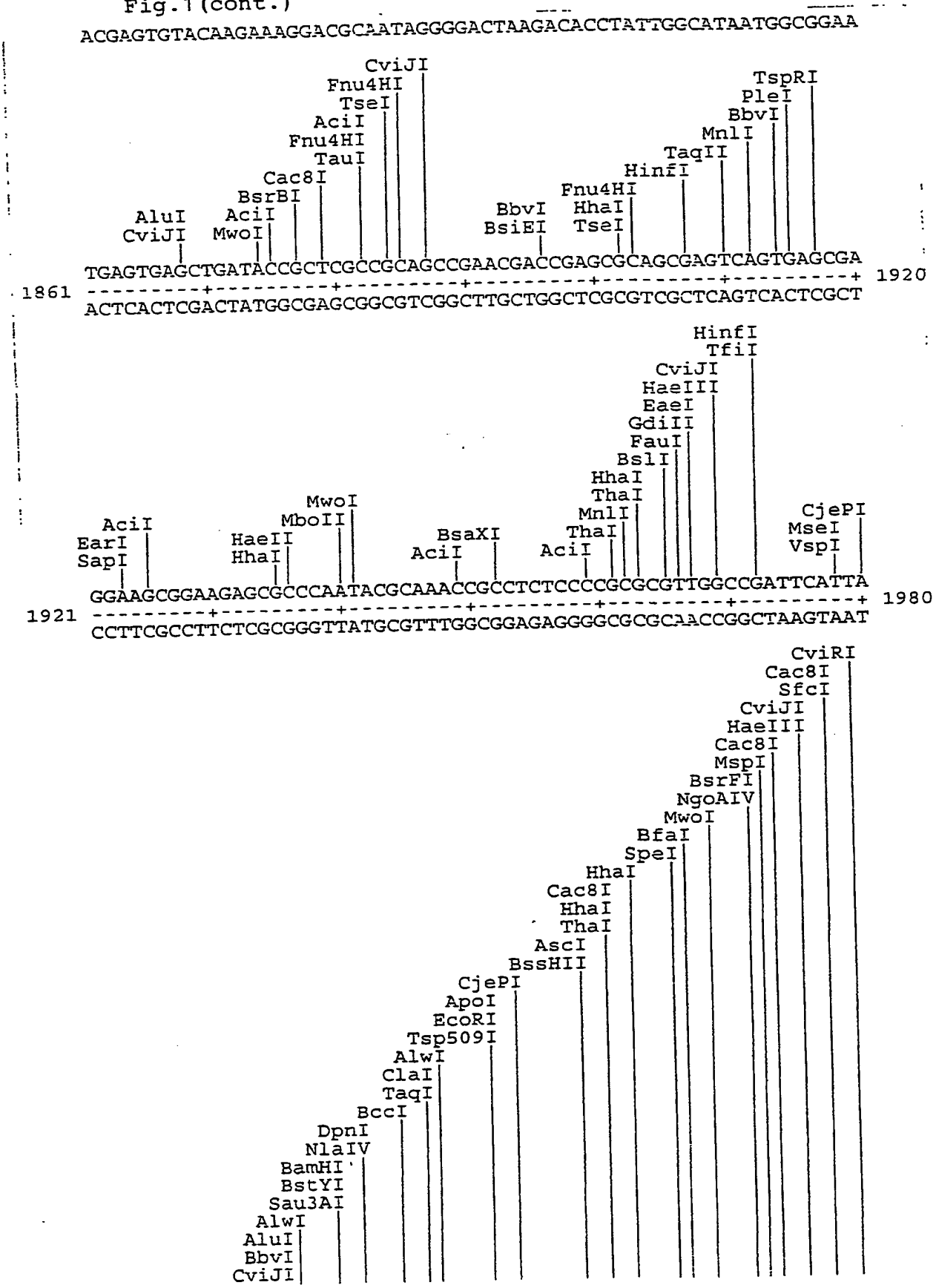
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Fig.1(cont.)

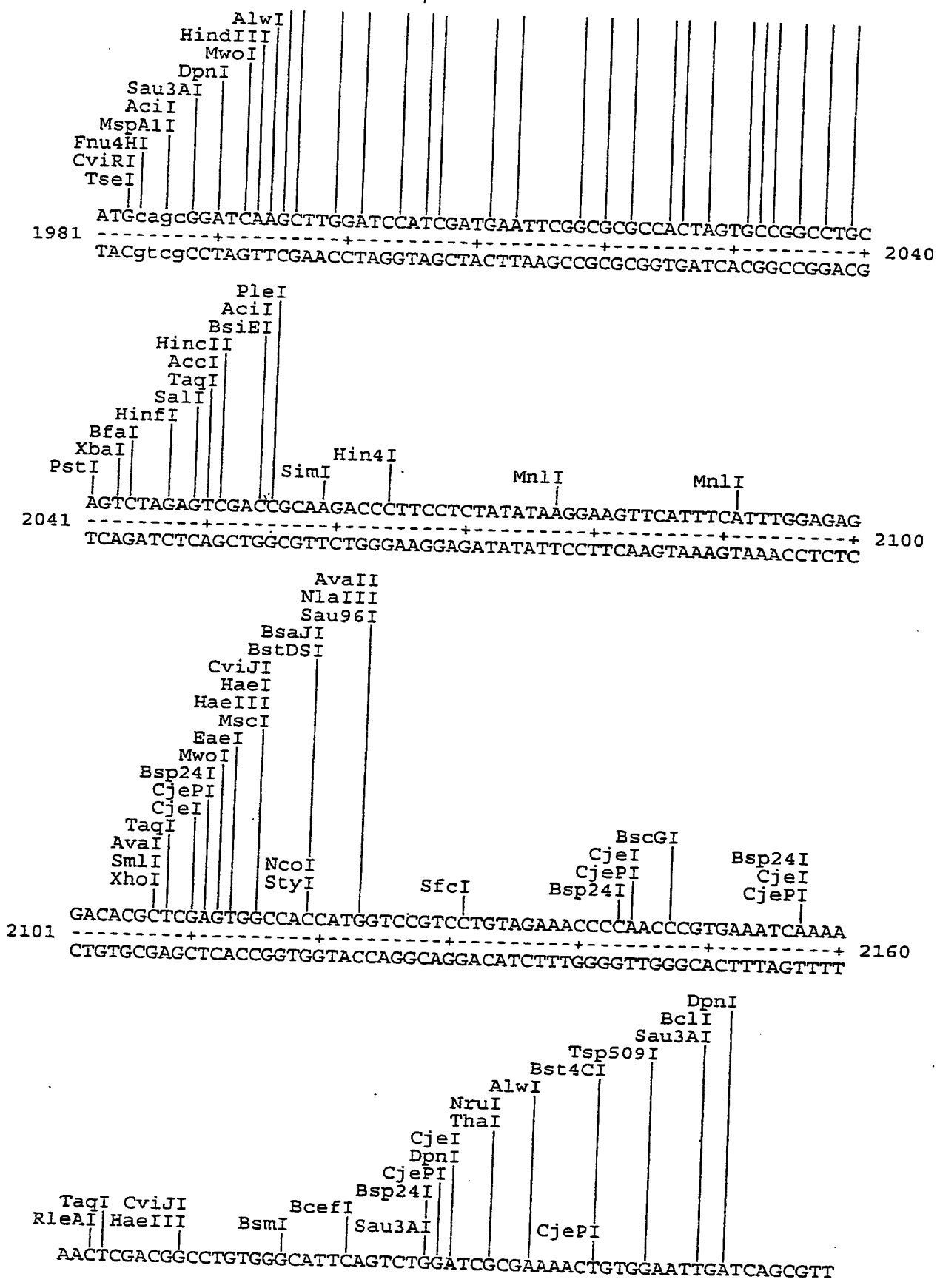
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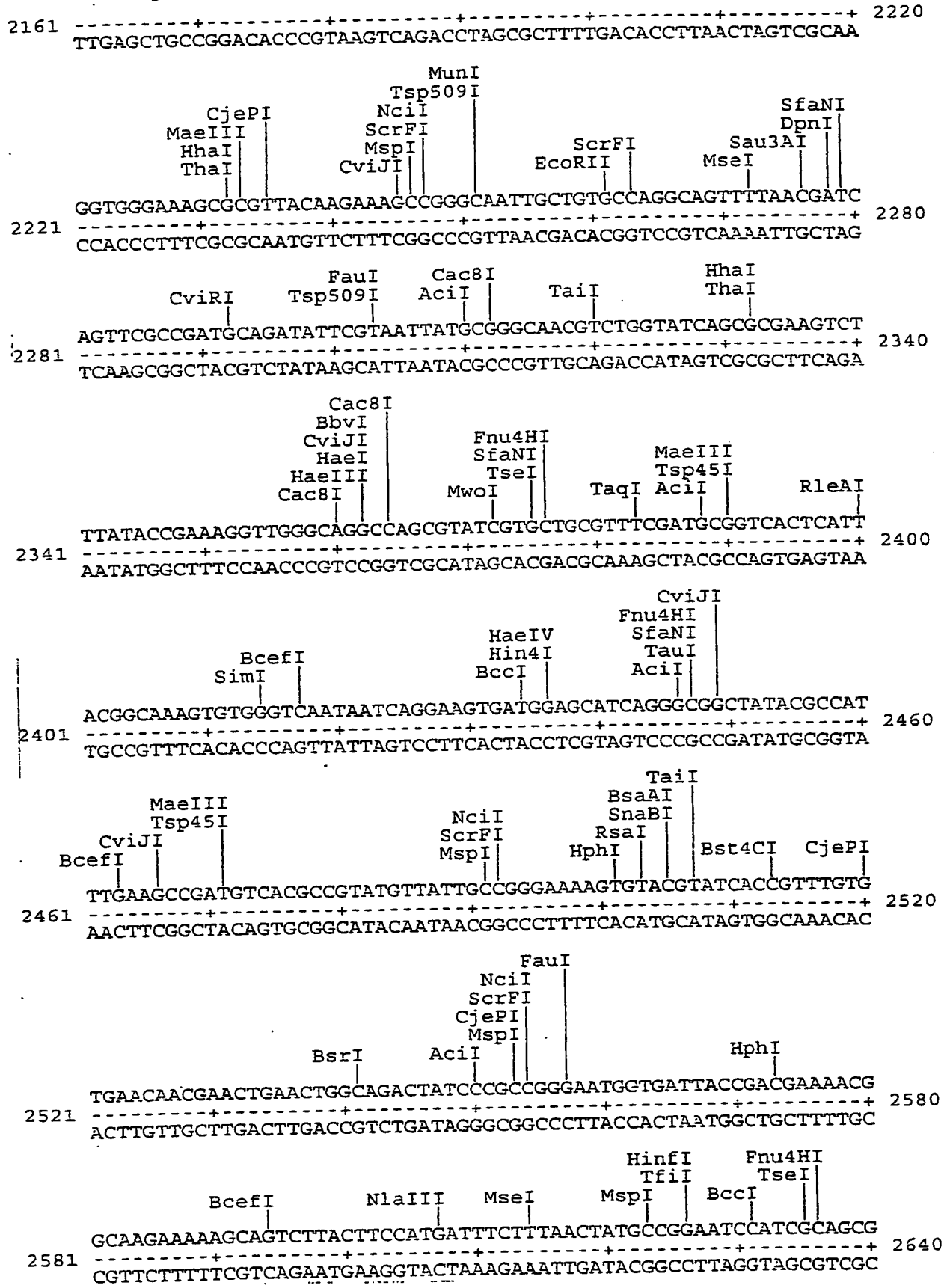
Fig.1(cont.) 8/20



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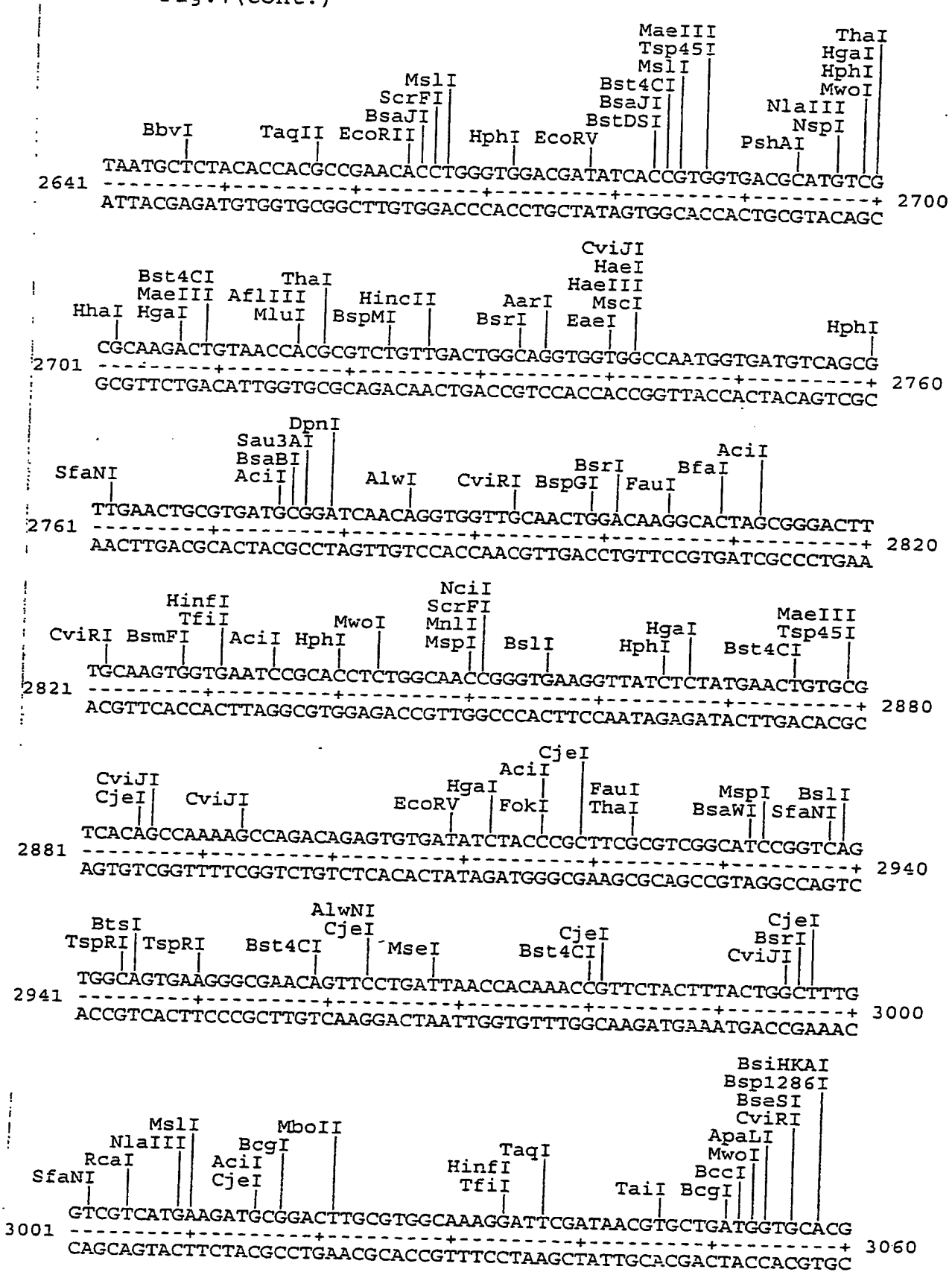
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TspRI
TspRI
Bst4CI
MseI
Bst4CI
BsrI
CviJI
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3000
BsiHKAI
Bsp1286I
BseSI
CviRI
MslI
MboII
ApaLI
NlaIII
BcgI
TaqI
RcaI
MwoI
AciI
HinfI
BccI
SfaNI
CjeI
TfiI
TaiI
BcgI
GTCGTCATGAAGATGCGGACTTGCGTGGCAAAGGATTCGATAACGTGCTGATGGTGCACG
3001
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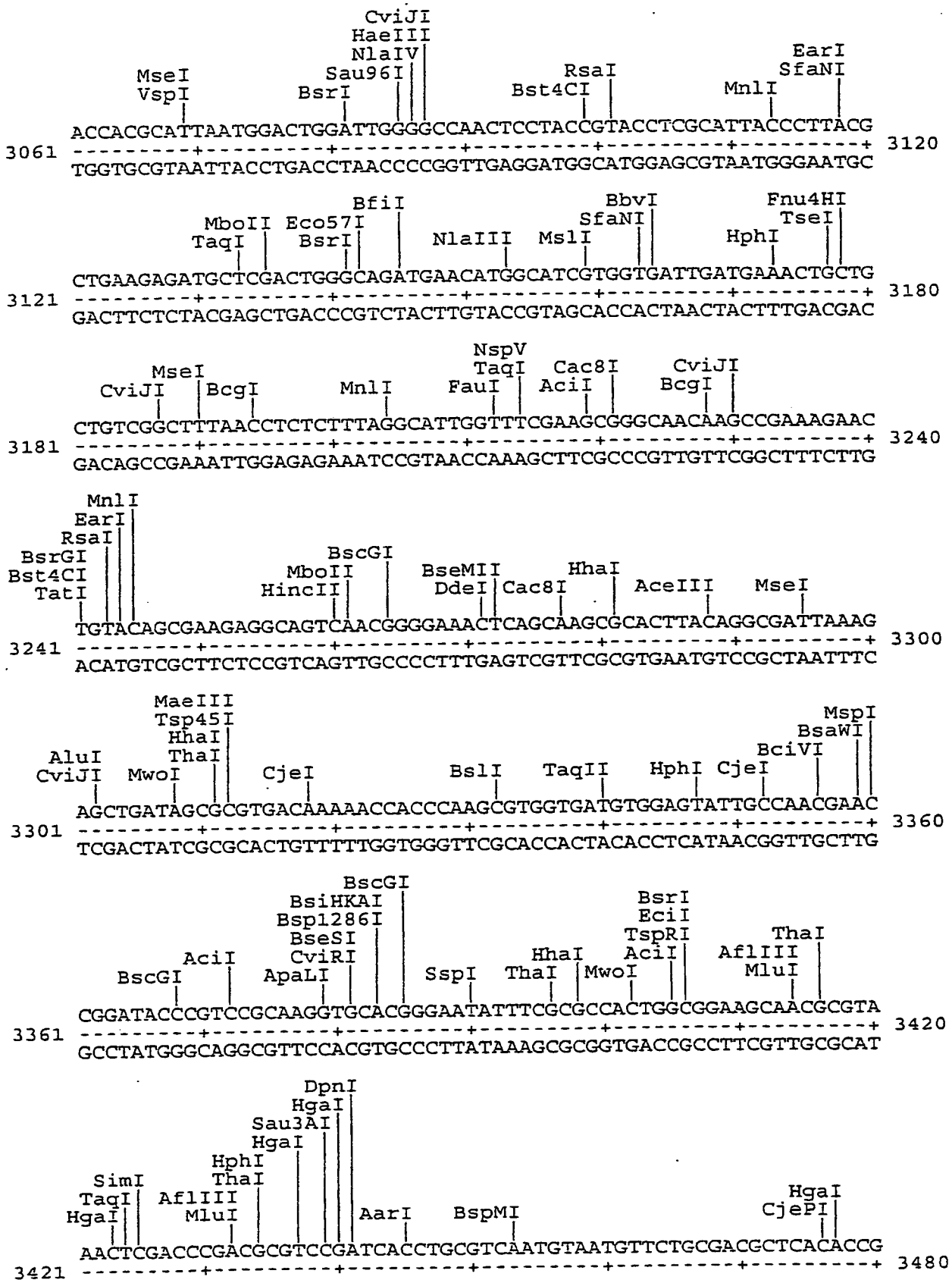
3060

Fig.1(cont.)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VspI
Sau96I
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RsaI
Bst4CI
EarI
SfaNI
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3180
NspV
TaqI
Cac8I
MseI
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BcgI
MnlI
FauI
AciI
CviJI
BcgI
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MnlI
EarI
RsaI
BscGI
BsrGI
MboII
BseMII
HhaI
Bst4CI
HincII
DdeI
Cac8I
AceIII
MseI
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MaeIII
Tsp45I
HhaI
MspI
BsaWI
AluI
ThaI
BciVI
CviJI
MwoI
CjeI
BslI
TaqII
HphI
CjeI
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BsiHKAI
Bsp1286I
BsrI
EciI
BseSI
TspRI
ThaI
AciI
CviRI
HhaI
AciI
AflIII
BscGI
ApaLI
SspI
ThaI
MwoI
MluI
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HgaI
Sau3AI
HgaI
HphI
ThaI
SimI
TaqI
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HgaI
MluI
AarI
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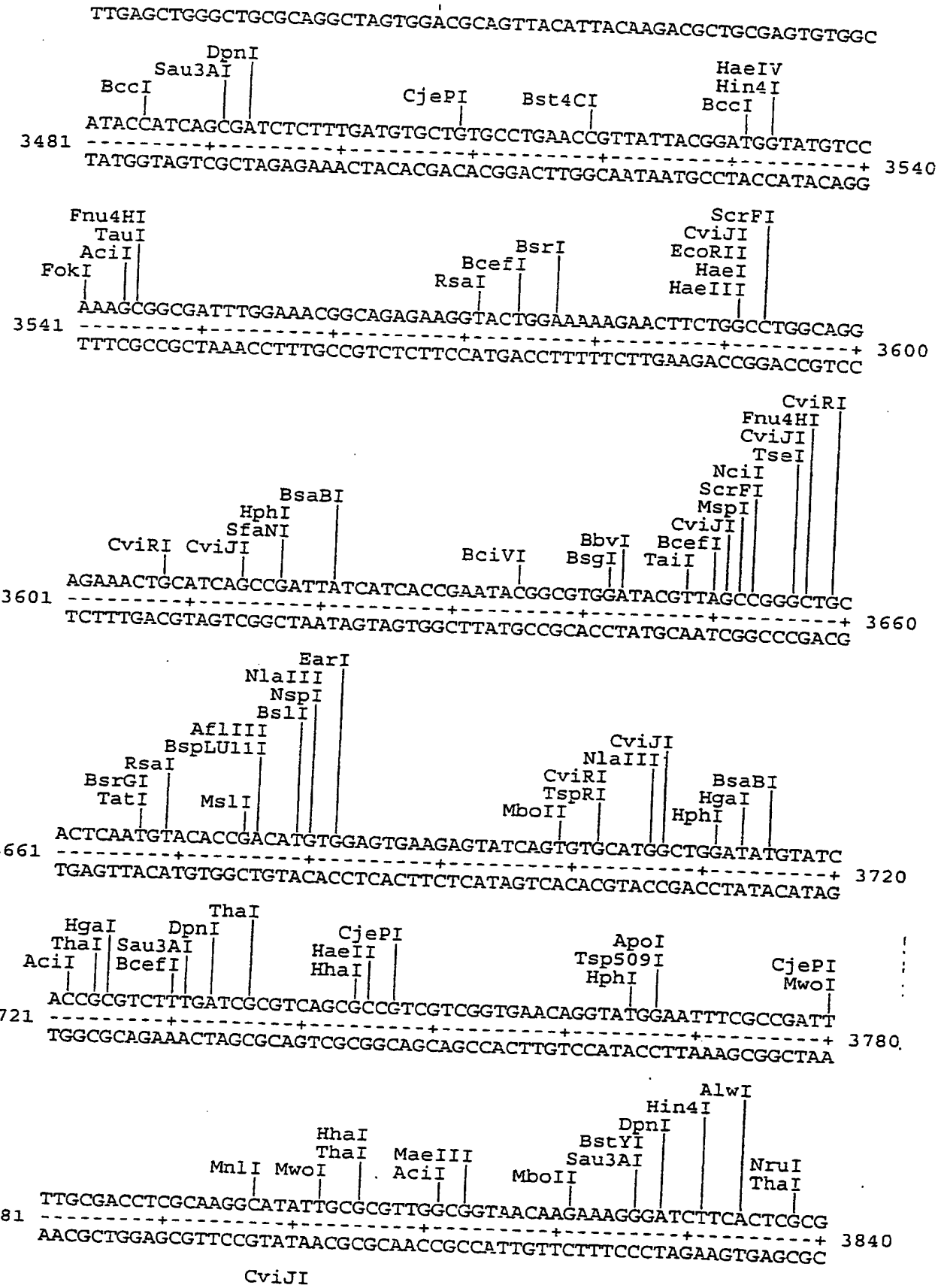
3421
3480

Fig.1(cont.)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FokI
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BcefI
RsaI
ScrFI
CviJI
EcoRII
HaeI
HaeIII
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3541
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3600
CviRI
Fnu4HI
CviJI
TseI
NciI
ScrFI
MspI
CviJI
BsaBI
HphI
SfaNI
CviRI
CviJI
BciVI
BbvI
BsgI
BcefI
TaiI
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3601
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NspI
BslI
AflIII
BspLU11I
RsaI
BsrGI
TatI
MslI
CviJI
NlaIII
CviRI
TspRI
MboII
BsaBI
HgaI
HphI
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3661
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HgaI
DpnI
CjePI
ThaI
Sau3AI
HaeII
ApoI
AciI
BcefI
HhaI
Tsp509I
CjePI
HphI
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HhaI
DpnI
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BstYI
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Sau3AI
Mnl1
MwoI
AciI
MboII
NruI
ThaI
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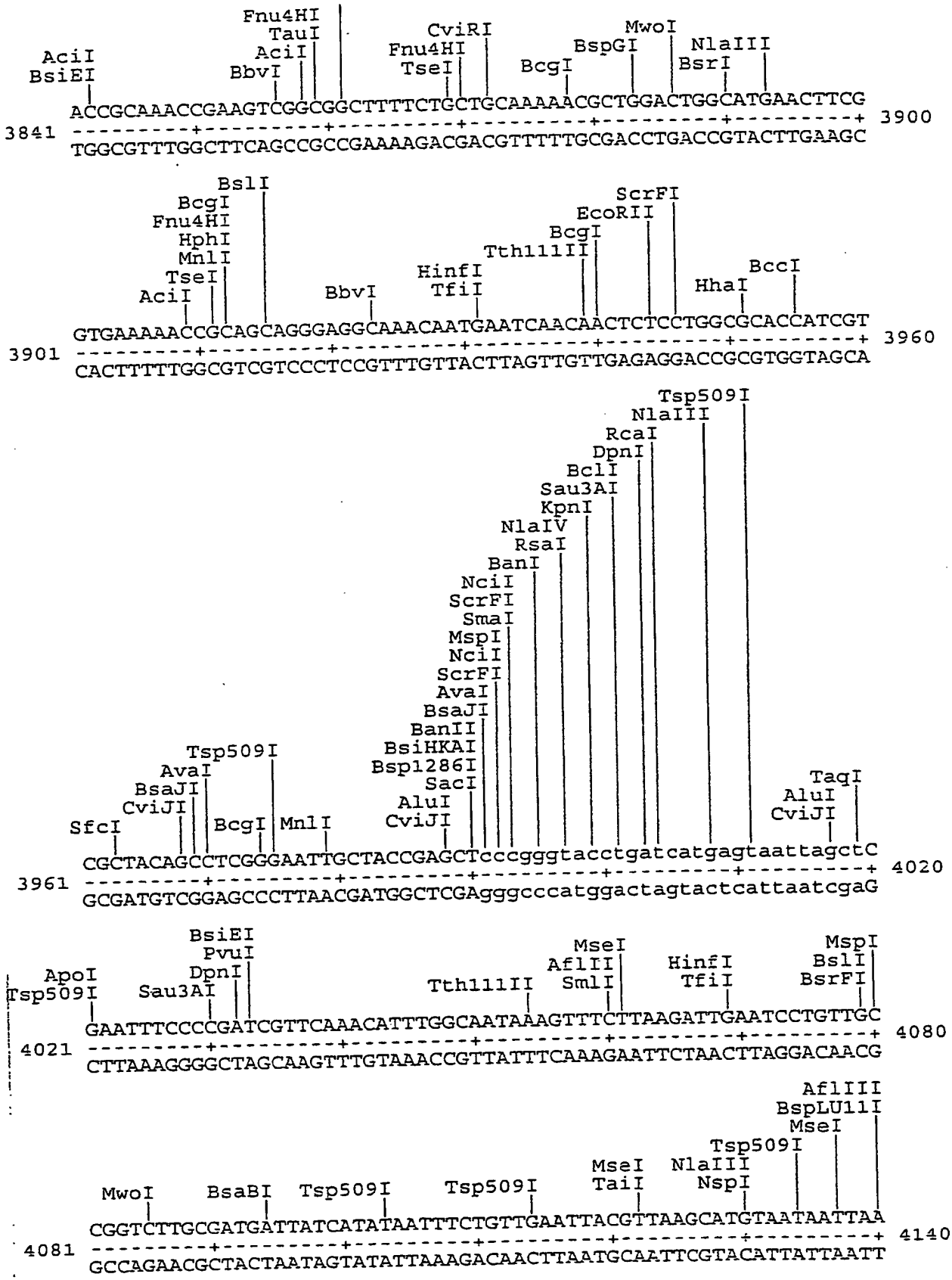
3840
CviJI

Fig.1(cont.)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TauI
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MwoI
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BsiEI
BbvI
TseI
BcgI
BsrI
ACCGCAAACCGAAGTCGGCGGCTTTTCTGCTGCAAAAACGCTGGACTGGCATGAACTTCG
3841 TGGCGTTTGGCTTCAGCCGCCGAAAAGACGACGTTTTTGCGACCTGACCGTACTTGAAGC 3900
BslI
BcgI
Fnu4HI
HphI
MnlI
TseI
AciI
BbvI
HinfI
TfiI
ScrFI
EcoRII
BcgI
Tth111II
BccI
HhaI
GTGAAAAACCGCAGCAGGGAGGCAAACAATGAATCAACAACTCTCCTGGCGCACCATCGT
3901 CACTTTTTGGCGTCGTCCCTCCGTTTGTTACTTAGTTGTTGAGAGGACCGCGTGGTAGCA 3960
Tsp509I
NlaIII
RcaI
DpnI
BclI
Sau3AI
KpnI
NlaIV
RsaI
BanI
NciI
ScrFI
SmaI
MspI
NciI
ScrFI
AvaI
BsaJI
BanII
BsiHKAI
Bsp1286I
SacI
AluI
CviJI
Tsp509I
AvaI
BsaJI
CviJI
SfcI
BcgI
MnlI
TaqI
AluI
CviJI
CGCTACAGCCTCGGGAATTGCTACCGAGCTcccgggtacctgatcatgagtaattagctC
3961 GCGATGTCGGAGCCCTTAACGATGGCTCGAgggcccatggactagtactcattaatcgaG 4020
BsiEI
PvuI
DpnI
ApoI
Tsp509I
Sau3AI
MseI
AflII
SmlI
Tth111II
HinfI
TfiI
MspI
BslI
BsrFI
GAATTTCCCCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGC
4021 CTTAAAGGGGCTAGCAAGTTTGTAAACCGTTATTTCAAAGAATTCTAACTTAGGACAACG 4080
AflIII
BspLU11I
MseI
Tsp509I
MseI
TaiI
NlaIII
NspI
MwoI
BsaBI
Tsp509I
Tsp509I
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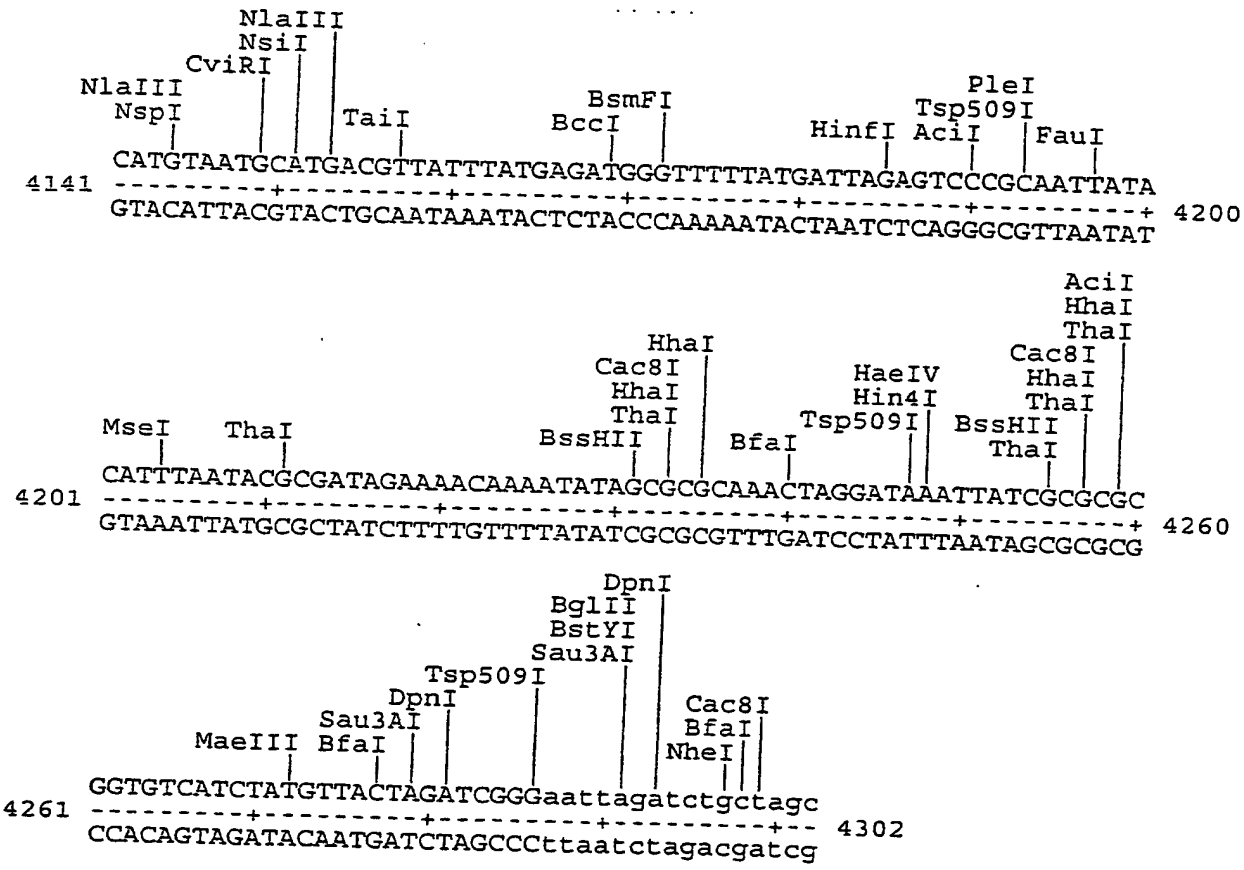
Fig.1(cont.)



Enzymes that do cut:

AarI	AccI	AceIII	AciI	AclI	AflII	AflIII	AhdI
AluI	AlwI	AlwNI	ApaLI	ApoI	AscI	AvaI	AvaII
BamHI	BanI	BanII	BbvI	BccI	Bce83I	BcefI	BcgI
BciVI	BclI	BfaI	BfiI	BglI	BglII	BpmI	BsaI
BsaAI	BsaBI	BsaHI	BsaJI	BsaWI	BsaXI	BscGI	BseMII
BseSI	BsgI	BsiEI	BsiHKAI	BslI	BsmI	BsmAI	BsmFI
Bsp24I	Bsp1286I	BspGI	BspLU11I	BspMI	BsrI	BsrBI	BsrDI
BsrFI	BsrGI	BssHII	BssSI	Bst4CI	BstDSI	BstYI	BtsI
Cac8I	CjeI	CjePI	ClaI	CviJI	CviRI	DdeI	DpnI
DraI	DrdI	EaeI	EarI	EciI	Eco57I	EcoRI	EcoRII
EcoRV	FauI	Fnu4HI	FokI	FspI	GdiII	HaeI	HaeII
HaeIII	HaeIV	HgaI	HgiEII	HhaI	Hin4I	HincII	HindIII
HinfI	HphI	KpnI	MaeIII	MboII	MluI	MmeI	MnlI
MscI	MseI	MslI	MspI	MspA1I	MunI	MwoI	NciI
NcoI	NgoAIV	NheI	NlaIII	NlaIV	NruI	NsiI	NspI
NspV	Pfl1108I	PleI	PshAI	PstI	PvuI	RcaI	RleAI
RsaI	SacI	SalI	SapI	Sau96I	Sau3AI	ScaI	ScrFI
SfaNI	SfcI	SimI	SmaI	SmlI	SnaBI	SpeI	SspI
StyI	TaiI	TaqI	TaqII	TatI	TauI	TfiI	ThaI
TseI	Tsp45I	Tsp509I	TspRI	Tth111II	UbaLI	VspI	XbaI
XhoI	XmnI						

Enzymes that do not cut:

AatII	AloI	ApaI	AvrII	BaeI	BbsI	BbvCI	BmgI
BplI	Bpu10I	Bpu1102I	BsbI	BseRI	BsmBI	BspEI	BstAPI
BstEII	BstXI	BstZ17I	Bsu36I	DraIII	DrdII	EagI	Eco47III
EcoNI	Eco0109I	FseI	HpaI	NarI	NdeI	NotI	PacI
Pf1MI	PinAI	PmeI	PmlI	Psp5II	PvuII	RsrII	SacII
SanDI	SbfI	SexAI	SfiI	SgfI	SgrAI	SphI	SrfI
Sse8647I	StuI	SunI	SwaI	Tth111I	XcmI		

14/20

Fig. 2

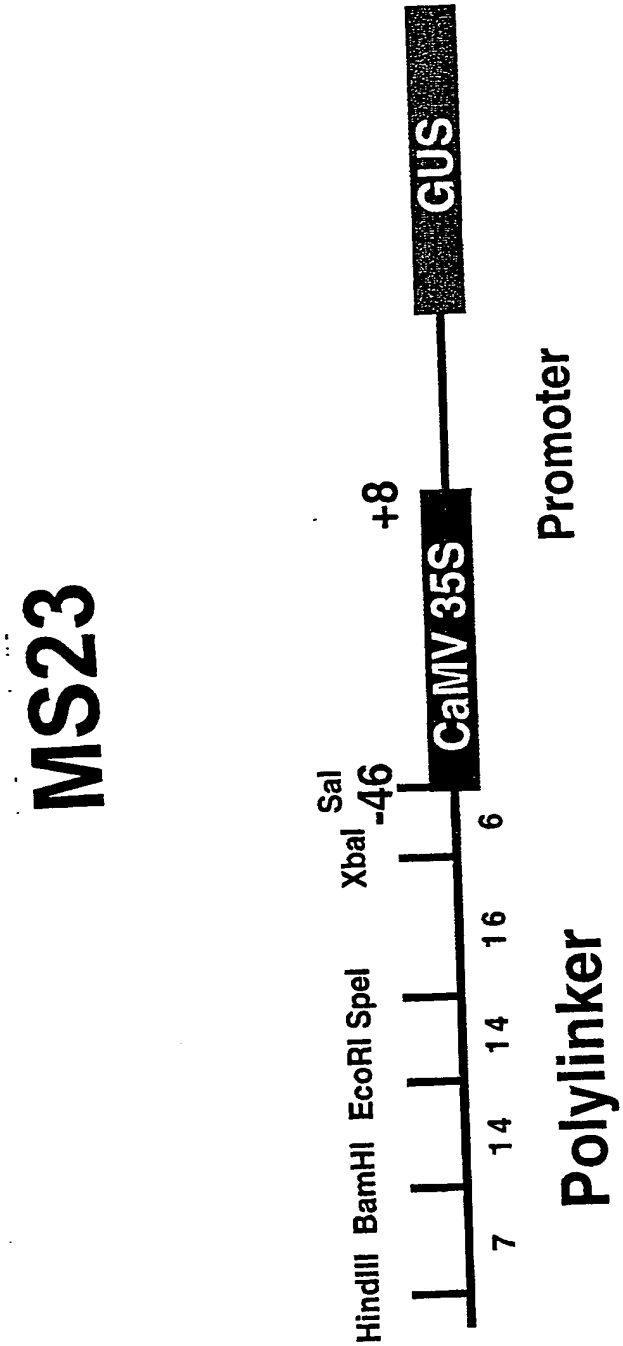
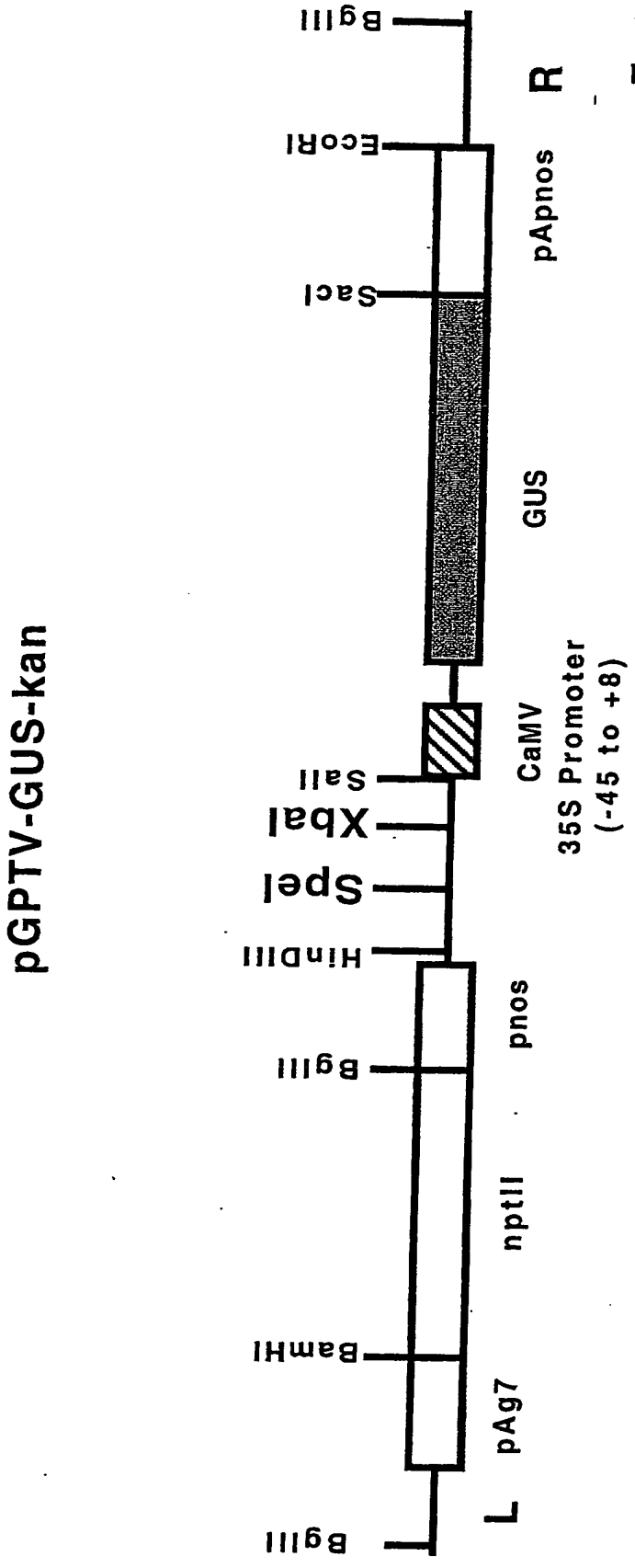

MS23
GUS
+8
CaMV 35S
Promoter
Sal
XbaI
-46
6
16
14
14
7
HindIII BamHI EcoRI SpeI
Polylinker

Fig. 3


pGPTV-GUS-kan
BglII
R
EcoRI
pAnos
SacI
GUS
CaMV
35S Promoter
(-45 to +8)
SalI
XbaI
SpeI
HinDIII
BglII
pnos
nptII
BamHI
pAg7
L
BglII

16/20

Fig. 4

fold induction
GUS activity (pmol/min/mg)
0
1000
4000
- elicitor
+ elicitor
2 ±1
5 ±1
7 ±2
6 ±3
1 ±1
50 ±19
14 ±5
construct
2s102
B109
C109
17s102
15s102
A109
18s102
dimer
reverse
AATTTGTTGACAGCCTTTTGGTCAAGCACTGACTTGG
TCAATATGTCAATGGTCAACATTCAAC
ØCAATATGTCAATGGTCAACATTCAAC
TCAATØTGTCAATGGTCAACATTCAAC
TCAATATGTØAATGGTCAACATTCAAC
TCAATATGTCAATGGTCAACATTCAAC
GTTCAATGTTGACCATTGACATATTGA

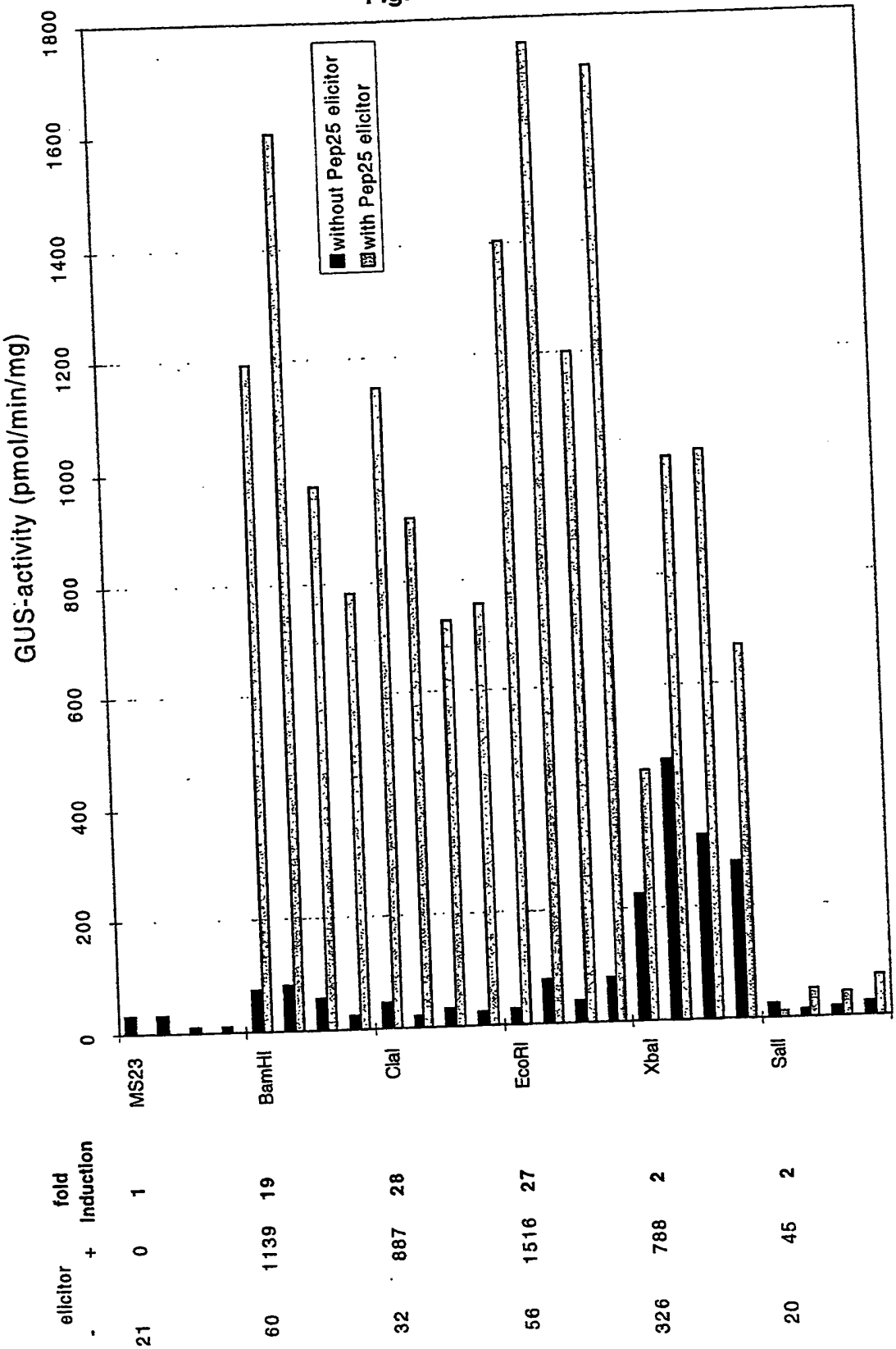
17/20

Fig. 5

SapI
GGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCagc
HindIII BamHI ClaI EcoRI SpeI XbaI SalI
GGATCAAGCTTGGATCCATCGATGAATTCGGCGCGCCACTAGTGCCGGCCTGCAGTCTAGAGTCGAC
CGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGCTCGAGTGGCCACCATGGTCCGTC...
35S minimal promoter
GUS

18/20

Fig. 6a


GUS-activity (pmol/min/mg)
0
200
400
600
800
1000
1200
1400
1600
1800
without Pep25 elicitor
with Pep25 elicitor
MS23
BamHI
ClaI
EcoRI
XbaI
SalI
elicitor - 21
elicitor + 0
fold Induction 1
elicitor - 60
elicitor + 1139
fold Induction 19
elicitor - 32
elicitor + 887
fold Induction 28
elicitor - 56
elicitor + 1516
fold Induction 27
elicitor - 326
elicitor + 788
fold Induction 2
elicitor - 20
elicitor + 45
fold Induction 2

19/20

Fig. 6b

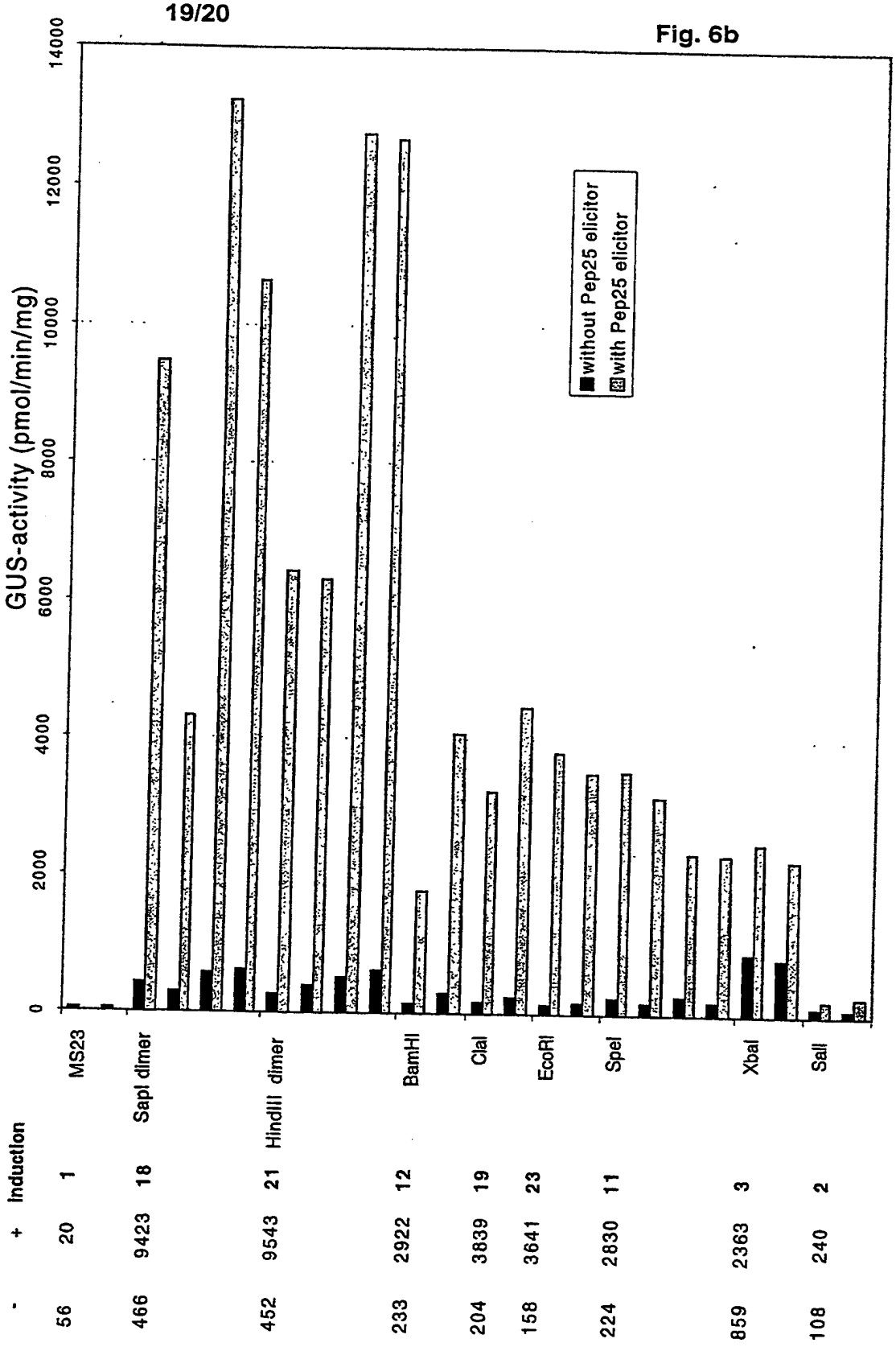

GUS-activity (pmol/min/mg)
0
2000
4000
6000
8000
10000
12000
14000
without Pep25 elicitor
with Pep25 elicitor
elicitor
fold
induction
- + induction
56 20 1 MS23
466 9423 18 SapI dimer
452 9543 21 HindIII dimer
233 2922 12 BamHI
204 3839 19 ClaI
158 3641 23 EcoRI
224 2830 11 SpeI
859 2363 3 XbaI
108 240 2 SalI

Fig. 7

Summary of Expression Characteristics

					Peronospora	
	Aerial parts	Roots	Wounding	Senescence	Incompatible	Compatible
4 x S	Medium	Medium	+	nt	+	+
4 x W1	Medium	Very high	+	nt	+	+
4 x GCC	Very high	Very high	+	nt	nt	nt
4 x D	-	-	-	+	+	+
4 x N	Medium	Medium	+	nt	+	+
4 x W_{Amy}	Low	Low	nt	nt	+	+
4 x W1	Medium	Medium	+	nt	+	+

Abstract

Described are synthetic promoters capable of mediating gene expression in plants upon pathogen infection. Furthermore, recombinant genes and vectors comprising said chimeric promoters as well as host cells transformed with such chimeric promoters, recombinant genes or vectors are provided. Additionally, diagnostic compositions and kits comprising such chimeric promoters, recombinant genes, vectors or cells are described. Provided are further methods for the identification of compounds being capable of activating or inhibiting genes that are specifically expressed in plants upon pathogen infection employing the above described means. Furthermore, transgenic plant cells, plant tissue and plants containing the above-described chimeric promoters, recombinant genes and vectors as well as the use of the aforementioned chimeric promoters, recombinant genes, vectors and/or compounds identified by the method of the invention in plant cell and tissue culture, plant breeding and/or agriculture are described.